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PLANT DESATURASESCOMPOSITIONS AND USESmw  
2/11/92

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This application is a continuation-in-part of PCT/US91/01746 filed March 14, 1991, ~~now pending~~, and a continuation-in-part of USSN 07/494,106 filed on March 16, 1990, now ~~pending~~ <sup>abandoned</sup>, and a continuation-in-part of USSN 07/615,784 filed on November 14, 1990, now ~~pending~~ <sup>abandoned</sup>, which is a continuation-in-part of USSN 07/567,373 filed on August 13, 1990, now abandoned, and a continuation-in-part of the above-referenced USSN 07/494,106.

15 Technical Field

The present invention is directed to desaturase enzymes relevant to fatty acid synthesis in plants, enzymes, amino acid and nucleic acid sequences and methods related thereto, and novel plant entities and/or oils and methods related thereto.

INTRODUCTIONBackground

Novel vegetable oils compositions and/or improved means to obtain or manipulate fatty acid compositions, from biosynthetic or natural plant sources, are needed. Depending

upon the intended oil use, various different oil compositions are desired. For example, edible oil sources containing the minimum possible amounts of saturates, palmitate (C16:0) and stearate (C18:0) saturated fatty acids, are desired for

5 dietary reasons and alternatives to current sources of highly saturated oil products, such as tropical oils, are also needed.

One means postulated to obtain such oils and/or modified fatty acid compositions is through the genetic engineering of

10 plants. The fatty acid composition of major oilseeds ordered here by palmitate content, is shown in Table I. With the exception of laurate (C12:0) sources of coconut endosperm and palm kernel, the common edible oils all basically consist of

15 (linolenate).

Table I

	<u>12:0</u>	<u>14:0</u>	<u>16:0</u>	<u>18:0</u>	<u>18:1</u>	<u>18:2</u>	<u>18:3</u>	<u>20:1</u>	<u>22:1</u>
			3	0.8	9.9	13.5	9.8	6.8	53.6
20			4.9	1.4	56.4	24.2	10.5		
		0.1	5.8	5.2	16	71.5	0.2		
			6.7	4.3	71.4	11.1	6.5		
			7.6	2	10.8	79.6			
	40.2	15.5	7.6	2.4	5.2	1.2			
25	50.9	18.4	8.7	1.9	14.6	1.2			
			15.3	3.8	20.7	55.8	9.4		
		1	23.4	2.5	17.9	54.2			
	0.1	1.2	46.8	3.8	37.6				
30									

However, in order to genetically engineer plants one must have in place the means to transfer genetic material to the plant in a stable and heritable manner. Additionally, one must have nucleic acid sequences capable of producing the desired phenotypic result, regulatory regions capable of directing the correct application of such sequences, and the like. Moreover, it should be appreciated that to produce a desired modified oils phenotype requires that the Fatty Acid Synthase (FAS) pathway of the plant is modified to the extent that the ratios of reactants are modulated or changed.

Higher plants appear to synthesize fatty acids via a common metabolic pathway in plant plastid organelles (i.e., chloroplasts, proplastids, or other related organelles) as part of the FAS complex. (By fatty acid is meant free fatty acids and acyl-fatty acid groups.) Outside of plastid organelles, fatty acids are incorporated into triacylglycerols (triglycerides) and used in plant membranes and in neutral lipids. In developing seeds, where oils are produced and stored as sources of energy for future use, FAS occurs in proplastids.

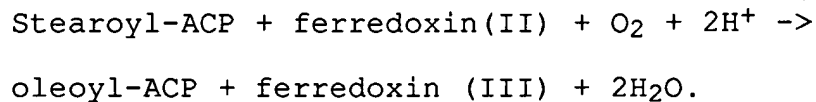
The production of fatty acids begins in the plastid with the reaction between Acyl Carrier Protein (ACP) and acetyl-CoA to produce acetyl-ACP. Through a sequence of reactions, the acetyl-ACP is elongated to 16- and 18- carbon fatty acids. The longest chain fatty acids produced by the FAS are 18 carbons long. ~~monounsaturated~~ fatty acids are also

~~cyclical~~ cyclical

~~monounsaturated~~  
monounsaturated

produced in the plastid through the action of a desaturase enzyme.

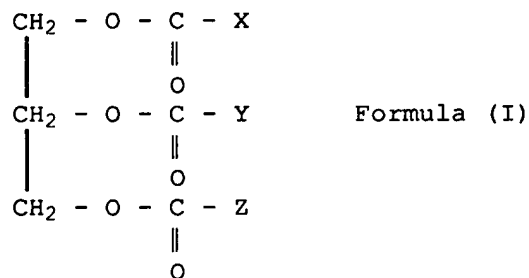
Common plant fatty acids, such as oleic, linoleic and  $\alpha$ -linolenic acids, are the result of sequential desaturation of stearate. The first desaturation step is the desaturation of stearyl-ACP (C18:0) to form oleoyl-ACP (C18:1) in a reaction often catalyzed by a  $\Delta$ -9 desaturase, also often referred to as a "stearyl-ACP desaturase" because of its high activity toward stearate the 18 carbon acyl-ACP. The desaturase enzyme functions to add a double bond at the ninth carbon in accordance with the following reaction (I):



$\Delta$ -9 desaturases have been studied in partially purified preparations from numerous plant species. Reports indicate that the protein is a dimer, perhaps a homodimer, displaying a molecular weight of 68 kD ( $\pm 8$  kD) by gel-filtration and a molecular weight of 36 kD by SDS-polyacrylamide gel electrophoresis.

In subsequent sequential steps for triglyceride production, polyunsaturated fatty acids may be produced. These desaturations occur outside of the plastid as a result of the action of membrane-bound enzymes. Additional double bonds are added at the twelve position carbon and thereafter, if added, at the 15 position carbon through the action of  $\Delta$ -12 desaturase and  $\Delta$ -15 desaturase, respectively.

The fatty acid composition of a plant cell is a reflection of the free fatty acid pool and the fatty acids (fatty acyl groups) incorporated into triglycerides. Thus, in a triglyceride molecule, represented as



X, Y, and Z each represent fatty acids which may be the same or different from one another. Various combinations of fatty acids in the different positions in the triglyceride will alter the properties of triglyceride. For example, if the fatty acyl groups are mostly saturated fatty acids, then the triglyceride will be solid at room temperature. In general, however, vegetable oils tend to be mixtures of different triglycerides. The triglyceride oil properties are therefore a result of the combination of triglycerides which make up the oil, which are in turn influenced by their respective fatty acid compositions.

For example, cocoa-butter has certain desirable qualities (mouth feel, sharp melting point, etc.) which are a function of its triglyceride composition. Cocoa-butter contains approximately 24.4% palmitate (16:0), 34.5% stearate (18:0), 39.1% oleate (18:1) and 2% linoleate (18:2). Thus, in cocoa butter, palmitate-oleate-stearate (POS) (i.e., X, Y and Z, respectively, in Formula I) comprises almost 50% of

triglyceride composition, with stearate-oleate-stearate (SOS) and palmitate-oleate-palmitate (POP) comprising the major portion of the balance at 39% and 16%, respectively, of the triglyceride composition.

5        Obtaining nucleic acid sequences capable of producing a phenotypic result in FAS, desaturation and/or incorporation of fatty acids into a glycerol backbone to produce an oil is subject to various obstacles including but not limited to the identification of metabolic factors of interest, choice and  
10        characterization of a protein source with useful kinetic properties, purification of the protein of interest to a level which will allow for its amino acid sequencing, utilizing amino acid sequence data to obtain a nucleic acid sequence capable of use as a probe to retrieve the desired  
15        DNA sequence, and the preparation of constructs, transformation and analysis of the resulting plants.

      Thus, the identification of enzyme targets and useful plant sources for nucleic acid sequences of such enzyme targets capable of modifying fatty acid compositions are  
20        needed. Ideally, an enzyme target will be amenable to one or more applications alone or in combination with other nucleic acid sequences relating to increased/decreased oil production, the ratio of saturated to unsaturated fatty acids in the fatty acid pool, and/or to novel oils compositions as  
25        a result of the modifications to the fatty acid pool. Once enzyme target(s) are identified and qualified, quantities of protein and purification protocols are needed for sequencing.

Ultimately, useful nucleic acid constructs having the necessary elements to provide a phenotypic modification and plants containing such constructs are needed.

## 5 Relevant Literature

A 200-fold purification of *Carthamus tinctorius* ("safflower") stearoyl-ACP desaturase was reported by McKeon & Stumpf in 1982, following the first publication of their protocol in 1981. McKeon, T. & Stumpf, P. *J.Biol.Chem.*

10 (1982) 257:12141-12147; McKeon, T. & Stumpf, P. *Methods in Enzymol.* (1981) 71:275-281.

## BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 provides amino acid sequence of fragments relating to *C. tinctorius* desaturase. Fragments F1 through  
 15 F11 are also provided in the sequence listing as SEQ ID NO: 1 through SEQ ID NO: 11, respectively. Each fragment represents a synthesis of sequence information from peptides originating from different digests which have been matched and aligned. In positions where there are two amino acids  
 20 indicated, the top one corresponds to that found in the translation of the cDNA; the lower one was detected either as a second signal at the same position of one of the sequenced peptides, or as a single unambiguous signal found in one or more of the overlapping peptides comprising the fragment.

25 Residues in F9 shown in lower case letters represent positions where the called sequence does not agree with that predicted from the cDNA, but where the amino acid assignment

is tentative because of the presence of a contaminating peptide. The standard one letter code for amino acid residues has been used. X represents a position where no signal was detectable, and which could be a modified residue.

5 F1 corresponds to the N-terminal sequence of the mature protein. The underlined region in F2 is the sequence used in designing PCR primers for probe synthesis.

Fig. 2 provides a cDNA sequence (SEQ ID NO: 12) and the corresponding translational peptide sequence (SEQ ID NO: 13) derived from *C. tinctorius* desaturase. The cDNA sequence includes both the plastid transit peptide encoding sequence and the sequence encoding the mature protein.

Fig. 3 provides cDNA sequence of *Ricinus communis* ("castor bean") desaturase. Fig. 3A provides preliminary partial cDNA sequence of a 1.7 kb clone of *R. communis* desaturase (SEQ ID NO: 14). The sequence is from the 5' end of the clone. Fig. 3B provides the complete cDNA sequence of the approximately 1.7 kb clone (SEQ ID NO: 15) and the corresponding translational peptide sequence (SEQ ID NO: 16).

20 Fig. 4 provides sequence of *Brassica campestris* ("rapeseed") desaturase. Fig. 4A represents partial DNA sequence of a 1.6 kb clone pCGN3235 (SEQ ID NO: 17), from the 5' end of the clone. Fig. 4B represents partial DNA sequence of a 1.2 kb clone, pCGN3236, from the 5' end of the clone (SEQ ID NO: 18). Initial sequence for the 3' ends of the two *B. campestris* desaturase clones indicates that pCGN3236 is a shorter cDNA for the same clone as pCGN3235. Fig. 4C

25

*Sub B1*



provides complete cDNA sequence of *B. campestris* desaturase above, pCGN3235 (SEQ ID NO: 19) and the corresponding translational peptide sequence (SEQ ID NO: 20).

Fig. 5 provides preliminary partial cDNA sequence of *Simmondsia chinensis* ("jojoba") desaturase (SEQ ID NO: 43). The translated amino acid sequence is also shown.

Fig. 6 shows the design of forward and reverse primers (SEQ ID NO: 21 through SEQ ID NO: 26) used in polymerase chain reaction (PCR) from the sequence of *C. tinctorius* desaturase peptide "Fragment F2" (SEQ ID NO: 2).

Fig. 7 provides maps of desaturase cDNA clones showing selected restriction enzyme sites. Fig. 7A represents a *C. tinctorius* clone, Fig. 7B represents a *R. communis* clone, and Fig. 7C represents a *B. campestris* clone.

Fig. 8 provides approximately 3.4 kb of genomic sequence of Bce4 (SEQ ID NO: 27).

Fig. 9 provides approximately 4 kb of genomic sequence of Bcg 4-4 ACP sequence (SEQ ID NO: 28).

Fig. 10 provides a restriction map of cloned  $\lambda$ CGN 1-2 showing the entire napin coding region sequence as well as extensive 5' upstream and 3' downstream sequences (SEQ ID NO: 29).

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#### SUMMARY OF THE INVENTION

By this invention, compositions and methods of use of plant desaturase enzymes, especially  $\Delta$ -9 desaturases, are

provided. Of special interest are methods and compositions of amino acids and nucleic acid sequences related to biologically active plant desaturases as well as sequences, especially nucleic acid sequences, which are to be used as probes, vectors for transformation or cloning intermediates. Biologically active sequences may be found in a sense or anti-sense orientation as to transcriptional regulatory regions found in various constructs.

A first aspect of this invention relates to *C. tinctorius*  $\Delta$ -9 desaturase substantially free of seed storage protein. Amino acid sequence of this desaturase is provided in Fig. 2 and as SEQ ID NO: 13.

DNA sequence of *C. tinctorius* desaturase gene (SEQ ID NO: 12) is provided, as well as DNA sequences of desaturase genes from a *Ricinus* (SEQ ID NO: 14 and SEQ ID NO: 15) a *Brassica* (SEQ ID NO: 17 through SEQ ID NO: 19) and a *Simmondsia* (SEQ ID NO: 43) plant.

In yet a different embodiment of this invention, plant desaturase cDNA of at least 10 nucleotides or preferably at least 20 nucleotides and more preferably still at least 50 nucleotides, known or homologously related to known  $\Delta$ -9 desaturase(s) is also provided. The cDNA encoding precursor desaturase or, alternatively, biologically active, mature desaturase is provided herein.

Methods to use nucleic acid sequences to obtain other plant desaturases are also provided. Thus, a plant desaturase may be obtained by the steps of contacting a

nucleic acid sequence probe comprising nucleotides of a known desaturase sequence and recovery of DNA sequences encoding plant desaturase having hybridized with the probe.

This invention also relates to methods for obtaining  
 5 plant  $\Delta$ -9 desaturase by contacting an antibody specific to a known desaturase, such as *C. tinctorius* stearyl-ACP desaturase, with a candidate plant stearyl-ACP desaturase under conditions conducive to the formation of an antigen:antibody immunocomplex and the recovery of the  
 10 candidate plant stearyl-ACP desaturase which reacts thereto.

In a further aspect of this invention DNA constructs comprising a first DNA sequence encoding a plant desaturase and a second DNA sequence which is not naturally found joined to said plant desaturase are provided. This invention also  
 15 relates to the presence of such constructs in host cells, especially plant host cells. In yet a different aspect, this invention relates to transgenic host cells which have an expressed desaturase therein.

Constructs of this invention may contain, in the 5' to  
 20 3' direction of transcription, a transcription initiation control regulatory region capable of promoting transcription in a host cell and a DNA sequence encoding plant desaturase. Transcription initiation control regulatory regions capable of expression in ~~prokaryotic~~ <sup>prokaryotic</sup> or eukaryotic host cells are  
 25 provided. Most preferred are transcription initiation control regions capable of expression in plant cells, and more preferred are transcription and translation initiation

regions preferentially expressed in plant cells during the period of lipid accumulation. The DNA sequence encoding plant desaturase of this invention may be found in either the sense or anti-sense orientation to the transcription

5 initiation control region.

Specific constructs, expression cassettes having in the 5' to 3' direction of transcription, a transcription and translation initiation control regulatory region comprising sequence immediately 5' to a structural gene preferentially  
10 expressed in plant seed during lipid accumulation, a DNA sequence encoding desaturase, and sequence 3' to the structural gene are also provided. The construct may preferably contain DNA sequences encoding plant desaturase obtainable (included obtained) from *Carthamus*, *Rininus*,  
15 *Brassica* or *Simmondsia*  $\Delta$ -9 desaturase genes. Transcription and translation initiation control regulatory regions are preferentially obtained from structural genes preferentially expressed in plant embryo tissue such as napin, seed-ACP or Bce-4 gene promoters.

20 By this invention, methods and constructs to inhibit the production of endogenous desaturase are also provided. For example, an anti-sense construct comprising, in the 5' to 3' direction of transcription, a transcription initiation control regulatory region functional in a plant cell, and an  
25 anti-sense DNA sequence encoding a portion of a plant  $\Delta$ -9 desaturase may be integrated into a plant host cell to decrease desaturase levels.

In yet a different embodiment, this invention is directed to a method of producing plant desaturase in a host cell comprising the steps of growing a host cell comprising an expression cassette, which would contain in the direction  
5 of transcription, a) a transcription and translation initiation region functional in said host cell, b) the DNA sequence encoding a plant desaturase in reading frame with said initiation region, and c) and a transcript termination region functional in said host cell, under conditions which  
10 will promote the expression of the plant desaturase. Cells containing a plant desaturase as a result of the production of the plant desaturase encoding sequence and also contemplated herein.

By this invention, a method of modifying fatty acid  
15 composition in a host plant cell from a given level of fatty acid saturation to a different level of fatty acid saturation is provided by growing a host plant cell having integrated into its genome a recombinant DNA sequence encoding a plant desaturase in either a sense or anti-sense orientation under  
20 control of regulatory elements functional in said plant cell during lipid accumulation under conditions which will promote the activity of said regulatory elements. Plant cells having such a modified level of fatty acid saturation are also contemplated hereunder. Oilseeds having such a modified  
25 level of fatty acid saturation and oils produced from such oilseeds are further provided.

### DETAILED DESCRIPTION OF THE INVENTION

A plant desaturase of this invention includes any sequence of amino acids, such as a protein, polypeptide, or peptide fragment, obtainable from a plant source which is  
5 capable of catalyzing the insertion of a first double bond into a fatty acyl-ACP moiety in a plant host cell, i.e., *in vivo*, or in a plant cell-like environment, i.e. *in vitro*. "A plant cell-like environment" means that any necessary conditions are available in an environment (i.e., such  
10 factors as temperatures, pH, lack of inhibiting substances) which will permit the enzyme to function. In particular, this invention relates to enzymes which add such a first double bond at the ninth carbon position in a fatty acyl-ACP chain. There may be similar plant desaturase enzymes of this  
15 invention with different specificities, such as the  $\Delta$ -12 desaturase of carrot.

Nucleotide sequences encoding desaturases may be obtained from natural sources or be partially or wholly artificially synthesized. They may directly correspond to a  
20 desaturase endogenous to a natural plant source or contain modified amino acid sequences, such as sequences which have been mutated, truncated, increased or the like. Desaturases may be obtained by a variety of methods, including but not limited to, partial or homogenous purification of plant  
25 extracts, protein modeling, nucleic acid probes, antibody preparations and sequence comparisons. Typically a plant

desaturase will be derived in whole or in part from a natural plant source.

Of special interest are  $\Delta$ -9 desaturases which are obtainable, including those which are obtained, from

5 *Carthamus*, *Ricinus*, *Simmondsia*, or *Brassica*, for example *C. tinctorius*, *R. communis*, *S. chinensis* and *B. campestris*, respectively, or from plant desaturases which are obtainable through the use of these sequences. "Obtainable" refers to those desaturases which have sufficiently similar sequences  
10 to that of the native sequences provided herein to provide a biologically active desaturase.

Once a DNA sequence which encodes a desaturase is obtained, it may be employed as a gene of interest in a nucleic acid construct or in probes in accordance with this  
15 invention. A desaturase may be produced in host cells for harvest or as a means of effecting a contact between the desaturase and its substrate. Constructs may be designed to produce desaturase in either prokaryotic or eukaryotic cells. Plant cells containing recombinant constructs encoding  
20 biologically active desaturase sequences, both expression and anti-sense constructs, as well as plants and cells containing modified levels of desaturase proteins are of special interest. For use in a plant cell, constructs may be designed which will effect an increase or a decrease in  
25 amount of desaturase enzyme available to a plant cell transformed with such a construct.

Where the target gene encodes an enzyme, such as a plant desaturase, which is already present in the host plant, there are inherent difficulties in analyzing mRNA, engineered protein or enzyme activity, and modified fatty acid composition or oil content in plant cells, especially in developing seeds; each of which can be evidence of biological activity. This is because the levels of the message, enzyme and various fatty acid species are changing rapidly during the stage where measurements are often made, and thus it can be difficult to discriminate between changes brought about by the presence of the foreign gene and those brought about by natural developmental changes in the seed. Where an expressed  $\Delta$ -9 desaturase DNA sequence is derived from a plant species heterologous to the plant host into which the sequence is introduced and has a distinguishable DNA sequence, it is often possible to specifically probe for expression of the foreign gene with oligonucleotides complimentary to unique sequences of the inserted DNA/RNA. And, if the foreign gene codes for a protein with slightly different protein sequence, it may be possible to obtain antibodies which recognize unique epitopes on the engineered protein. Such antibodies can be obtained by mixing the antiserum to the foreign protein with extract from the host plant, or with extracts containing the host plant enzyme. For example, one can isolate antibodies uniquely specific to a *C. tinctorius*  $\Delta$ -9 desaturase by mixing antiserum to the desaturase with an extract containing a *Brassica*  $\Delta$ -9



desaturase. Such an approach will allow the detection of *C. tinctorius* desaturase in *Brassica* plants transformed with the *C. tinctorius* desaturase gene. In plants expressing an endogenous gene in an antisense orientation, the situation is slightly different. In this case, there are no specific reagents to measure expression of a foreign protein.

However, one is attempting to measure a decrease in an enzyme activity that normally is increasing during development.

This makes detection of expression a simpler matter. In the final seed maturation phase, enzyme activities encoded by genes affecting oil composition usually disappear and cannot be detected in final mature seed. Analysis of the fatty acid content may be ~~performed~~ <sup>performed</sup> by any manner known to those skilled in the art, including gas chromatography, for example.

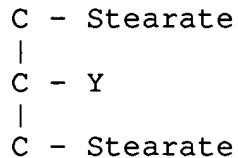
By increasing the amount of desaturase available in the plant cell, an increased percentage of unsaturated fatty acids may be provided; by decreasing the amount of desaturase, an increased percentage of saturated fatty acids may be provided. (Modifications in the pool of fatty acids available for incorporation into triglycerides may likewise affect the composition of oils in the plant cell.) Thus, an increased expression of desaturase in a plant cell may result in increased proportion of fatty acids, such as one or more of palmitoleate (C16:1), oleate (C18:1), linoleate (C18:2) and linolenate (C18:3) are expected. In rapeseed, increased desaturase expression lowers stearate and total saturates. Of special interest is the production of triglycerides having

increased levels of oleate. Using anti-sense technology, alternatively, a decrease in the amount of desaturase available to the plant cell is expected, resulting in a higher percentage of saturates such as one or more of laurate (C12:0), myristate (C14:0), palmitate (C16:0), stearate (C18:0), arachidate (C20:0), behemate (C22:0) and lignocerate (C24:0). In rapeseed reduced desaturase results in increased stearate levels and total saturates. Of special interest is the production of triglycerides having increased levels of stearate or palmitate and stearate. In addition, the production of a variety of ranges of such saturates is desired. Thus, plant cells having lower and higher levels of stearate fatty acids are contemplated. For example, fatty acid compositions, including oils, having a 10% level of stearate as well as compositions designed to have up to an appropriate 60% level of stearate or other such modified fatty acid(s) composition are contemplated.

Oils with increased percentages of stearate, especially rapeseed triglyceride oils, are provided herein. Increased stearate percentages (by weight) ranging from native up to 25 fold are described. By manipulation of various aspects of the DNA constructs (e.g., choice of promoters, number of copies, etc.) and traditional breeding methods, one skilled in the art may achieve even greater levels of stearate. By combination of the plant desaturase sequence in combination with other DNA sequences, a variety of other fatty acid

compositions and triglycerides can be achieved in rapeseed and other plant species.

Oilseed containing stearate rich fatty acids having the majority incorporated into triglyceride oils will contain a certain percentage of triglycerides of the following formula:



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Wherein Y is an unsaturated fatty acid. In a certain triglycerides, Y shall be oleate. Triglyceride oils with stearate-unsaturate-stearate (S-U-S) and/or stearate-oleate-stearate (S-O-S) may be novel oils compositions, particularly in oilseed varieties which naturally contain low stearate levels. Such triglyceride oils may find special application in the production of non-hydrogenated margarines, for example. Edible oils having naturally low stearate levels include canola (rapeseed), sunflower, peanut, safflower, coconut and oil palm, (See, Table I.)

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The modification of fatty acid compositions may also affect the fluidity of plant membranes. Different lipid concentrations have been observed in cold-hardened plants, for example. By this invention, one may be capable of introducing traits which will lend to chill tolerance. Constitutive or temperature inducible transcription initiation regulatory control regions may have special applications for such uses.

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Other applications for use of cells or plants producing desaturase may also be found. For example, potential herbicidal agents selective for plant desaturase may be obtained through screening to ultimately provide environmentally safe herbicide products. The plant desaturase can also be used in conjunction with chloroplast lysates to enhance the production and/or modify the composition of the fatty acids prepared *in vitro*. The desaturase can also be used for studying the mechanism of fatty acid formation in plants and bacteria. For these applications, constitutive promoters may find the best use.

Constructs which contain elements to provide the transcription and translation of a nucleic acid sequence of interest in a host cell are "expression cassettes".

Depending upon the host, the regulatory regions will vary, including regions from structural genes from viruses, plasmid or chromosomal genes, or the like. For expression in prokaryotic or eukaryotic microorganisms, particularly unicellular hosts, a wide variety of constitutive or regulatable promoters may be employed. Among transcriptional initiation regions which have been described are regions from bacterial and yeast hosts, such as *E. coli*, *B. subtilis*, *Saccharomyces cerevisiae*, including genes such as  $\beta$ -galactosidase, T7 polymerase, trp E and the like.

A recombinant construct for expression of desaturase in a plant cell ("expression cassette") will include, in the 5' to 3' direction of transcription, a transcription and a

translation initiation control regulatory region (the transcriptional and translational initiation regions together often also known as a "promoter") functional in a plant cell, a nucleic acid sequence encoding a plant desaturase, and a transcription termination region. Numerous transcription initiation regions are available which provide for a wide variety of constitutive or regulatable, e.g., inducible, transcription of the desaturase structural gene. Among transcriptional initiation regions used for plants are such regions associated with cauliflower mosaic viruses (35S, 19S), and structural genes such as for nopaline synthase or mannopine synthase or napin and ACP promoters, etc. The transcription/translation initiation regions corresponding to such structural genes are found immediately 5' upstream to the respective start codons. Thus, depending upon the intended use, different promoters may be desired.

Of special interest in this invention are the use of promoters which are capable of preferentially expressing the desaturase in seed tissue, in particular, at early stages of seed oil formation. Examples of such seed-specific promoters include the region immediately 5' upstream of napin or seed ACP genes, such as described in co-pending USSN 147,781, and the Bce-4 gene such as described in co-pending USSN 494,722. Alternatively, the use of the 5' regulatory region associated with an endogenous plant desaturase structural gene and/or the transcription termination regions found immediately 3' downstream to the gene, may often be desired.

In addition, for some applications, use of more than one promoter may be desired. For example, one may design a dual promoter expression cassette each promoter having a desaturase sequence under its regulatory control. For example, the combination of an ACP and napin cassette could be useful for increased production of desaturase in a seed-specific fashion over a longer period of time and/or to exert a greater production of desaturase and hence have a greater effect on the cell than either individually.

To decrease the amount of desaturase found in a plant host cell, anti-sense constructs may be prepared and then inserted into the plant cell. By "anti-sense" is meant a DNA sequence in the 5' to 3' direction of transcription in relation to the transcription initiation region, which encodes a sequence complementary to the sequence of a native desaturase. It is preferred that an anti-sense plant desaturase sequence be complementary to a plant desaturase gene indigenous to the plant host. Sequences found in an anti-sense orientation may be found in constructs providing for transcription or transcription and translation of the DNA sequence encoding the desaturase, including expression cassettes. Constructs having more than one desaturase sequence under the control of more than one promoter or transcription initiation region may also be employed with desaturase constructs. Various transcription initiation regions may be employed. One of ordinary skill in the art can readily determine suitable regulatory regions. Care may

be necessary in selecting transcription initiation regions to avoid decreasing desaturase activity in plant cells other than oilseed tissues. Any transcription initiation region capable of directing expression in a plant host which causes  
5 initiation of adequate levels of transcription selectively in storage tissues during seed development for example, should be sufficient. As such, seed specific promoters may be desired. Other manners of decreasing the amount of endogenous plant desaturase, such as ribozymes or the  
10 screening of plant cells transformed with constructs for rare events containing sense sequences which in fact act to decrease desaturase expression, are also contemplated herein. Other analogous methods may be applied by those of ordinary skill in the art.

15 By careful selection of plants, transformants having particular oils profiles may be obtained. This may in part depend upon the qualities of the transcription initiation region(s) employed or may be a result of culling transformation events to exploit the variabilities of  
20 expression observed.

For some applications, modified fatty acid compositions may be detected in developing seeds, whereas in other instances, such as for analysis of oil profile, detection of fatty acid modifications occurring later in the FAS pathway,  
25 or for detection of minor modifications to the fatty acid composition, analysis of fatty acid or oil from mature seeds may be preferred. Furthermore, analysis of oil and/or fatty

acid content of individual seeds may be desirable, especially in detection of oil modification in the segregating T1 seed populations. As used herein, T1 indicates the plant and seed arising from transformation/regeneration protocols described  
5 herein. T2 indicates plants and seeds generated from the transgenic T1 seed.

In order to obtain the nucleic acid sequences encoding *C. tinctorius* desaturase via protein analysis, a protein preparation free of a major albumin-type contaminant is  
10 required. As demonstrated more fully in the Examples, the protocols of McKeon and Stumpf, *supra*, result in a preparation contaminated with a seed storage protein. Removal of the protein contaminant may be effected by application of a reverse-phase HPLC, or alternatively, by  
15 application of a reduction and alkylation step followed by electrophoresis and blotting, for example. Other purification methods may be employed as well, now that the presence of the contaminant is confirmed and various properties thereof described. Once the purified desaturase  
20 is obtained it may be used to obtain the corresponding amino acid and/or nucleic acid sequences thereto in accordance with methods familiar to those skilled in the art. Approximately 90% of the total amino acid sequence of the *C. tinctorius* desaturase is provided in Fig. 1 and in SEQ ID NOS: 1-11.  
25 The desaturase produced in accordance with the subject invention can be used in preparing antibodies for assays for detecting plant desaturase from other sources.



A nucleic acid sequence of this invention may include genomic or cDNA sequence and mRNA. A cDNA sequence may or may not contain pre-processing sequences, such as transit peptide sequences. Transit peptide sequences facilitate the delivery of the protein to a given organelle and are cleaved from the amino acid moiety upon entry into the organelle, releasing the "mature" sequence.

In Fig. 2 and SEQ ID NO: 13, the sequence of the *C. tinctorius* desaturase precursor protein is provided; both the transit peptide and mature protein sequence are shown. Also provided in this invention are cDNA sequences relating to *R. communis* desaturase (Fig.3 and SEQ ID NOS: 14-15), *B. campestris* desaturase (Fig. 4 and SEQ ID NOS: 17-19) and *S. chinesis* (Fig. 5 and SEQ ID NOS: 43).

The use of the precursor cDNA sequence is preferred in desaturase expression cassettes. In addition, desaturase transit peptide sequences may be employed to translocate other proteins of interest to plastid organelles for a variety of uses, including the modulation of other enzymes related to the FAS pathway. See, European Patent Application Publication No. 189,707.

As described in more detail below, the complete genomic sequence of a desaturase may be obtained by the screening of a genomic library with a desaturase cDNA probe and isolating those sequences which regulate expression in seed tissue. In this manner, the transcription, translation initiation regions and/or transcript termination regions of the

desaturase may be obtained for use in a variety of DNA constructs, with or without the respective desaturase structural gene.

Other nucleic acid sequences "homologous" or "related" to DNA sequences encoding other desaturases are also provided. "Homologous" or "related" includes those nucleic acid sequences which are identical or conservatively substituted as compared to the exemplified *C. tinctorius*, *R. communis*, *S. chinesis* or *B. campestris* desaturase sequences of this invention or a plant desaturase which has in turn been obtained from a plant desaturase of this invention. By conservatively substituted is meant that codon substitutions encode the same amino acid, as a result of the degeneracy of the DNA code, or that a different amino acid having similar properties to the original amino acid is substituted. One skilled in the art will readily recognize that antibody preparations, nucleic acid probes (DNA and RNA) sequences encoding and the like may be prepared and used to screen and recover desaturase from other plant sources. Typically, nucleic acid probes are labeled to allow detection, preferably with radioactivity although enzymes or other methods may also be used. For immunological screening methods, antibody preparations either monoclonal or polyclonal are utilized. Polyclonal antibodies, although less specific, typically are more useful in gene isolation. For detection, the antibody is labeled using radioactivity or any one of a variety of second antibody/enzyme conjugate

systems that are commercially available. Examples of some of the available antibody detection systems are described by Oberfilder (*Focus* (1989) BRL Life Technologies, Inc., 11:1-5).

5       A "homologous" or "related" nucleic acid sequence will show at least about 60% homology, and more preferably at least about 70% homology, between the known desaturase sequence and the desired candidate plant desaturase of interest, excluding any deletions which may be present.

10      Homology is determined upon comparison of sequence information, nucleic acid or amino acid, or through hybridization reactions. Amino acid sequences are considered homologous by as little as 25% sequence identity between the two complete mature proteins. (See generally, Doolittle,  
15      R.F., of URFS and ORFS, University Science Books, CA, 1986.)

          Oligonucleotide probes can be considerably shorter than the entire sequence, but should be at least about 10, preferably at least about 15, more preferably at least 20 nucleotides in length. When shorter length regions are used  
20      for comparison, a higher degree of sequence identity is required than for longer sequences. Shorter probes are often particularly useful for polymerase chain reactions (PCR), especially when highly conserved sequences can be identified. (See, Gould, et al., *PNAS USA* (1989) 86:1934-1938.) Longer  
25      oligonucleotides are also useful, up to the full length of the gene encoding the polypeptide of interest. When longer nucleic acid fragments are employed (>100 bp) as probes,

especially when using complete or large cDNA sequences, one would screen with low stringencies (for example 40-50°C below the melting temperature of the probe) in order to obtain signal from the target sample with 20-50% deviation, i.e., homologous sequences. (See, Beltz, et al., *Methods in Enzymology* (1983) 100:266-285.) Both DNA and RNA probes can be used.

A genomic library prepared from the plant source of interest may be probed with conserved sequences from a known desaturase to identify homologously related sequences. Use of the entire cDNA may be employed if shorter probe sequences are not identified. Positive clones are then analyzed by restriction enzyme digestion and/or sequencing. When a genomic library is used, one or more sequences may be identified providing both the coding region, as well as the transcriptional regulatory elements of the desaturase gene from such plant source. In this general manner, one or more sequences may be identified providing both the coding region, as well as the transcriptional regulatory elements of the desaturase gene from such plant source.

In use, probes are typically labeled in a detectable manner (for example with  $^{32}\text{P}$ -labeled or biotinylated nucleotides) and are incubated with single-stranded DNA or RNA from the plant source in which the gene is sought, although unlabeled oligonucleotides are also useful. Hybridization is detected by means of the label after single-stranded and double-stranded (hybridized) DNA or DNA/RNA have

been separated, typically using nitrocellulose paper or nylon membranes. Hybridization techniques suitable for use with oligonucleotides are well known to those skilled in the art.

Thus, plant desaturase genes may be isolated by various

5 techniques from any convenient plant. Plant desaturase of developing seed obtained from other oilseed plants, such as soybean, coconut, oilseed rape, sunflower, oil palm, peanut, cocoa, cotton, corn and the like are desired as well as from non-traditional oil sources, including but not limited to  
10 spinach chloroplast, avocado mesocarp, cuphea, California Bay, cucumber, carrot, meadowfoam, *Oenothera* and *Euglena gracillis*.

Once the desired plant desaturase sequence is obtained, it may be manipulated in a variety of ways. Where the

15 sequence involves non-coding flanking regions, the flanking regions may be subjected to resection, mutagenesis, etc.

Thus, transitions, transversions, deletions, and insertions may be performed on the naturally occurring sequence. In

addition, all or part of the sequence may be synthesized,

20 where one or more codons may be modified to provide for a modified amino acid sequence, or one or more codon mutations

may be introduced to provide for a convenient restriction site or other purpose involved with construction or

expression. The structural gene may be further modified by

25 employing synthetic adapters, linkers to introduce one or more convenient restriction sites, or the like.

Recombinant constructs containing a nucleic acid sequence encoding a desaturase of this invention may be combined with other, i.e. "heterologous," DNA sequences in a variety of ways. By heterologous DNA sequences is meant any  
5 DNA sequence which is not naturally found joined to the native desaturase, including combinations of DNA sequences from the same plant of the plant desaturase which are not naturally found joined together. In a preferred embodiment, the DNA sequence encoding a plant desaturase is combined in a  
10 DNA construct having, in the 5' to 3' direction of transcription, a transcription initiation control region capable of promoting transcription in a host cell, and a DNA sequence encoding a desaturase in either a sense or anti-sense orientation. As described in more detail elsewhere, a  
15 variety of regulatory control regions containing transcriptional or transcriptional and translational regions may be employed, including all or part of the non-coding regions of the plant desaturase.

The open reading frame coding for the plant desaturase  
20 or functional fragment thereof will be joined at its 5' end to a transcription initiation regulatory control region. In some instances, such as modulation of plant desaturase via a desaturase in an anti-sense orientation, a transcription initiation region or transcription/translation initiation  
25 region may be used. In embodiments wherein the expression of the desaturase protein is desired in a plant host, a transcription/ translation initiation regulatory region, is

needed. Additionally, modified promoters, i.e., having transcription initiation regions derived from one gene source and translation initiation regions derived from a different gene source or enhanced promoters, such as double 35S CaMV  
5 promoters, may be employed for some applications.

As described above, of particular interest are those 5' upstream non-coding regions which are obtained from genes regulated during seed maturation, particularly those preferentially expressed in plant embryo tissue, such as ACP-  
10 and napin-derived transcription initiation control regions. Such regulatory regions are active during lipid accumulation and therefore offer potential for greater control and/or effectiveness to modify the production of plant desaturase and/or modification of the fatty acid composition.

15 Especially of interest are transcription initiation regions which are preferentially expressed in seed tissue, i.e., which are undetectable in other plant parts. For this purpose, the transcript initiation region of acyl carrier protein isolated from *B. campestris* seed and designated as  
20 "Bcg 4-4" and an unidentified gene isolated from *B. campestris* seed and designated as "Bce-4" are also of substantial interest.

Briefly, Bce4 is found in immature embryo tissue at least as early as 11 days after anthesis (flowering), peaking  
25 about 6 to 8 days later or 17-19 days post-anthesis, and becoming undetectable by 35 days post-anthesis. The timing of expression of the Bce4 gene closely follows that of lipid

accumulation in seed tissue. Bce4 is primarily detected in seed embryo tissue and to a lesser extent found in the seed coat. Bce4 has not been detected in other plant tissues tested, root, stem and leaves.

5        Approximately 3.4 kb genomic sequence of Bce4 is provided in Fig. 8 and as SEQ ID NO: 27, including about 1 kb 5' to the structural gene, about 0.3 kb of the Bce4 coding gene sequence, and about 2.1 kb of the non-coding regulatory 3' sequence. Bce4 transcript initiation regions will contain  
10    at least 1 kb and more preferably about 5 to about 7.5 kb of sequence immediately 5' to the Bce4 structural gene.

      The Bcg 4-4 ACP message presents a similar expression profile to that of Bce4 and, therefore, also corresponds to lipid accumulation in the seed tissue. Bcg 4-4 is not found  
15    in the seed coat and may show some differences in expression level, as compared to Bce4, when the Bcg 4-4 5' non-coding sequence is used to regulate transcription or transcription and translation of a plant stearyl-ACP desaturase of this invention. Genomic sequence of Bcg 4-4 is provided in Fig. 9  
20    and as SEQ ID NO: 28, including about 1.5 kb 5' to the structural gene, about 1.2 kb of the Bcg 4-4 (ACP) structural gene sequence, and about 1.3 kb of the non-coding regulatory 3' sequence.

      The napin 1-2 message is found in early seed development  
25    and thus, also offers regulatory regions which can offer preferential transcriptional regulation of a desired DNA sequence of interest such as the plant desaturase DNA



sequence of this invention during lipid accumulation. Napins are one of the two classes of storage proteins synthesized in developing *Brassica* embryos (Bhatty, et al., *Can J. Biochem.* (1968) 46:1191-1197) and have been used to direct tissue-specific expression when reintroduced into the *Brassica* genome (Radke, et al., *Theor. Appl. Genet.* (1988) 75:685-694). Genomic sequence of napin 1-2 is provided in Fig. 10 and as SEQ ID NO: 29, including about 1.7 kb 5' to the structural gene and about 1.3 kb of the non-coding regulatory 3' sequence

Regulatory transcript termination regions may be provided in DNA constructs of this invention as well. Transcript termination regions may be provided by the DNA sequence encoding the plant desaturase or a convenient transcription termination region derived from a different gene source, especially the transcript termination region which is naturally associated with the transcript initiation region. The transcript termination region will contain at least about 1 kb, preferably about 3 kb of sequence 3' to the structural gene from which the termination region is derived.

In developing the DNA construct, the various components of the construct or fragments thereof will normally be inserted into a convenient cloning vector which is capable of replication in a bacterial host, e.g., *E. coli*. Numerous vectors exist that have been described in the literature. After each cloning, the plasmid may be isolated and subjected to further manipulation, such as restriction, insertion of

new fragments, ligation, deletion, insertion, resection,  
etc., so as to tailor the components of the desired sequence.  
Once the construct has been completed, it may then be  
transferred to an appropriate vector for further manipulation  
5 in accordance with the manner of transformation of the host  
cell.

Normally, included with the DNA construct will be a  
structural gene having the necessary regulatory regions for  
expression in a host and providing for selection of  
10 transformant cells. The gene may provide for resistance to a  
cytotoxic agent, e.g. antibiotic, heavy metal, toxin, etc.,  
complementation providing prototrophy to an auxotrophic host,  
viral immunity or the like. Depending upon the number of  
different host species into which the expression construct or  
15 components thereof are introduced, one or more markers may be  
employed, where different conditions for selection are used  
for the different hosts.

The manner in which the DNA construct is introduced into  
the plant host is not critical to this invention. Any method  
20 which provides for efficient transformation may be employed.  
Various methods for plant cell transformation include the use  
of Ti- or Ri-plasmids, microinjection, electroporation,  
liposome fusion, DNA bombardment or the like. In many  
instances, it will be desirable to have the construct  
25 bordered on one or both sides by T-DNA, particularly having  
the left and right borders, more particularly the right  
border. This is particularly useful when the construct uses

*A. tumefaciens* or *A. rhizogenes* as a mode for transformation, although the T-DNA borders may find use with other modes of transformation.

Where *Agrobacterium* is used for plant cell transformation, a vector may be used which may be introduced into the *Agrobacterium* host for homologous recombination with T-DNA or the Ti- or Ri-plasmid present in the *Agrobacterium* host. The Ti- or Ri-plasmid containing the T-DNA for recombination may be armed (capable of causing gall formation) or disarmed (incapable of causing gall formation), the latter being permissible, so long as the *vir* genes are present in the transformed *Agrobacterium* host. The armed plasmid can give a mixture of normal plant cell and gall.

A preferred method for the use of *Agrobacterium* as the vehicle for transformation of plant cells employs a vector having a broad host range replication system, at least one T-DNA boundary and the DNA sequence or sequences of interest. Commonly used vectors include pRK2 or derivatives thereof. See, for example, Ditta et al., *PNAS USA*, (1980) 77:7347-7351 and EPA 0 120 515, which are incorporated herein by reference. Normally, the vector will be free of genes coding for opines, oncogenes and *vir*-genes. Included with the expression construct and the T-DNA will be one or more markers, which allow for selection of transformed *Agrobacterium* and transformed plant cells. A number of markers have been developed for use with plant cells, such as resistance to chloramphenicol, the aminoglycoside G418,

hygromycin, or the like. The particular marker employed is not essential to this invention, one or another marker being preferred depending on the particular host and the manner of construction.

5       The expression constructs may be employed with a wide variety of plant life, particularly plant life involved in the production of vegetable oils. These plants include, but are not limited to rapeseed, sunflower, *C. tinctorius*, cotton, *Cuphea*, peanut, soybean, oil palm and corn. Anti-  
10 sense constructs may be employed in such plants which share complementarity between the endogenous sequence and the anti-sense desaturase. Of special interest is the use of an anti-sense construct having a *B. campestris* desaturase in rapeseed, including *B. campestris* and *B. napus*.

15       For transformation of plant cells using *Agrobacterium*, explants may be combined and incubated with the transformed *Agrobacterium* for sufficient time for transformation, the bacteria killed, and the plant cells cultured in an appropriate selective medium. Once callus forms, shoot  
20 formation can be encouraged by employing the appropriate plant hormones in accordance with known methods and the shoots transferred to rooting medium for regeneration of plants. The plants may then be grown to seed and the seed used to establish repetitive generations and for isolation of  
25 vegetable oils compositions. A variety of stable genetic lines having fixed levels of saturation may be obtained and integrated into a traditional breeding program. Hemizygous

and heterozygous lines or homozygous lines may demonstrate different useful properties for oil production and/or breeding. For example, saturation levels may be increased up to 2-fold by the development of homozygous plants as compared with heterozygous (including hemizygous) plants.

The invention now being generally described, it will be more readily understood by reference to the following examples which are included for purposes of illustration only and are not intended to limit the present invention.

## EXAMPLES

### MATERIALS

Commercially available biological chemicals and chromatographic materials, including BSA, catalase (twice ~~crystallized~~ <sup>crystallized</sup> from bovine liver), spinach ferredoxin, ferredoxin-NADP<sup>+</sup> oxidoreductase (spinach leaf), NADPH, unlabeled fatty acids, DEAE-cellulose (Whatman DE-52) CNBr-activated Sepharose 4B, and octyl-Sepharose, and Reactive Blue Agarose are from Sigma (St. Louis, MO). Triethylamine, trichloroacetic acid, guanidine-HCl, and hydrazine-hydrate are also from Sigma. Proteolytic enzymes, including endoproteinases lysC, gluC, and aspN are sequencing grade enzymes obtained from Boehringer Mannheim (Indianapolis, IN). Organic solvents, including acetone, acetonitrile, methanol, ether and petroleum ether are purchased from J.T. Baker (Phillipsburg, NJ); concentrated acids and sodium sulfate are also from J.T. Baker (Phillipsburg, NJ). HPLC grade

acetonitrile and trifluoroacetic acid (TFA) are obtained from Burdick and Jackson (Muskegon, MI), and from Applied Biosystems (Foster City, CA), respectively. Radiochemicals, including [9,10(n)-<sup>3</sup>H] oleic acid (10mCi/μmol) and [<sup>3</sup>H]-iodoacetic acid (64Ci/mol) are from New England Nuclear (Boston, MA). Phenacyl-8 Reagent (bromoacetophenone with a crown ether catalyst) used to prepare phenacyl esters of the fatty acids for analysis are from Pierce (Rockford, IL). C18 reversed-phase thin-layer chromatography plates are from Whatman (Clifton, NJ).

Acyl carrier protein (ACP) and acyl-ACP synthase are isolated from *E. coli* strain K-12 as described by Rock and Cronan (Rock and Cronan, *Methods in Enzymol* (1981) 71:341-351 and Rock et al., *Methods in Enzymol.* (1981) 72:397-403). The *E. coli* is obtainable from Grain Processing (Iowa) as frozen late-logarithmic phase cells.

[9,10(n)-<sup>3</sup>H]stearic acid is synthesized by reduction of [9,10(n)-<sup>3</sup>H]oleic acid with hydrazine hydrate essentially as described by Johnson and Gurr (*Lipids* (1971) 6:78-84). [9,10(n)-<sup>3</sup>H]oleic acid (2 mCi), supplemented with 5.58mg unlabeled oleic acid to give a final specific radioactivity of 100mCi/mmol, is dissolved in 2ml of acetonitrile, acidified with 40μl of glacial acetic acid, and heated to 55°C. Reduction is initiated with 100μl of 60% (w/w) hydrazine hydrate; oxygen is bubbled through the mixture continuously. After each hour acetonitrile is added to bring the volume back to 2ml and an additional 100μl of hydrazine

hydrate is added. At the end of 5 hr. the reaction is stopped by addition of 3ml of 2M HCl. The reaction products are extracted with three 3ml aliquots of petroleum ether and the combined ether extracts are washed with water, dried over sodium sulfate and evaporated to dryness. The dried reaction products are redissolved in 1.0ml acetonitrile and stored at -20°C. The distribution of fatty acid products in a 15µl aliquot is determined by preparation of phenacyl esters, which are then analyzed by thin layer chromatography on C-18 reverse phase plates developed with methanol:water:95:5 (v/v). Usually reduction to [9,10(n)-<sup>3</sup>H]stearic acid is greater than 90%, a small amount of unreacted oleic acid may remain. The analysis is used to establish fraction of the total radioactivity that is present as stearate, and thereby to determine the exact substrate concentration in the enzyme assay.

Acyl-ACP substrates, including [9,10(n)-<sup>3</sup>H] stearoyl-ACP are prepared and purified by the enzymatic synthesis procedure of Rock, Garwin, and Cronan (*Methods in Enzymol.* (1981) 72:397-403).

Acyl carrier protein was covalently bound to Sepharose 4B by reaction of highly purified ACP with CNBr-activated Sepharose 4B as described by McKeon and Stumpf (*J. Biol. Chem.* (1982) 257:12141-12147).

Example 1

In this example, an initial purification of *C. tinctorius* (safflower) desaturase, following the method of McKeon and Stumpf (*J. Biol. Chem.* (1982) 257:12141-12142), is described.

Assay: In each of the following steps, the presence of the enzyme is detected radiometrically by measuring enzyme-catalyzed release of tritium from [9,10(n)-<sup>3</sup>H]stearoyl-ACP. Preparation of this substrate is described in "Materials" above.

The assay is performed by mixing 150μl water, 5ml dithiothreitol (100mM, freshly prepared in water), 10μl bovine serum albumin (10mg/ml in water), 15μl NADPH (25mM, freshly prepared in 0.1M Tricine-HCl, pH 8.2), 25μl spinach ferredoxin (2mg/ml Sigma Type III in water), 3μl NADPH:ferredoxin oxidoreductase (2.5 units/ml from Sigma), and 1 μl bovine liver catalase (800,000 units/ml from Sigma); after 10 min at room temperature, this mixture is added to a 13x100 mm screw-cap test tube containing 250μl sodium 1,4-piperazinediethanesulfonate (0.1M, pH 6.0). Finally, 10μl of the sample to be assayed is added and the reaction is started by adding 30μl of the substrate, [9,10(n)-<sup>3</sup>H]stearoyl-ACP (100μCi/μmol, 10μM in 0.1M sodium 1,4-piperazinediethanesulfonate, pH 5.8). After sealing with a cap, the reaction is allowed to proceed for 10 min. while shaking at 23°C. The reaction is terminated by addition of 1.2ml of 5.8% ~~trichloroacetic~~ trichloroacetic acid and the resulting

1 trichloroacetic



precipitated acyl-ACP's are removed by centrifugation. The tritium released into the aqueous supernatant by the desaturase reaction is measured by liquid scintillation spectrometry. One unit of activity is defined as the amount  
5 of enzyme required to convert 1 $\mu$ mol of stearoyl-ACP to oleoyl-ACP, or to release 4 $\mu$ g-atoms of  $^3\text{H}$  per minute.

*Source tissue:* Developing *C. tinctorius* seeds from greenhouse grown plants are harvested between 16 and 18 days after flowering, frozen in liquid nitrogen and stored at -  
10  $70^{\circ}\text{C}$  until extracted.

*Acetone Powder:* Approximately 50g of frozen seeds are ground in liquid nitrogen and sieved to remove large seed coat pieces to provide a fine embryo powder. The powder is washed with acetone on a Buchner funnel until all yellow  
15 color is absent from the filtrate. The powder is then air dried and further processed as described below, or may be stored frozen for at least a year at  $-70^{\circ}\text{C}$  without loss of enzyme activity.

*Acetone Powder Extract:* The dried acetone powder is  
20 weighed and triturated with ten times its weight of 20mM potassium phosphate, pH 6.8; the mixture is then centrifuged at 12,000 x g for 20 minutes and decanted through a layer of Miracloth (Calbiochem, La Jolla, CA).

*Ion Exchange Chromatography:* The acetone powder extract  
25 is then applied to a DEAE-cellulose column (Whatman DE-52) (1.5 x 12 cm) equilibrated with 20mM potassium phosphate, pH

6.8. The pass-through and a wash with one column-volume (20ml) of buffer are pooled.

*Affinity Chromatography:* An affinity matrix for purification of the desaturase is prepared by reacting highly purified *E. coli* ACP, with CNBr-activated Sepharose 4B (Sigma). ACP (120mg) is reduced by treatment with 1mM dithiothreitol for 30 min on ice, and then desalted on Sephadex G-10 (Pharmacia) equilibrated with 0.1M sodium bicarbonate, pH 6.0. The treated ACP (20 ml, 6 mg/ml) is then mixed with 20ml of CNBr-activated Sepharose 4B swollen in 0.1M sodium bicarbonate, pH 7.0, and the mixture is allowed to stand at 4°C for one day. The gel suspension is then centrifuged, washed once with 0.1M sodium bicarbonate, pH 7.0, and then treated with 40ml 0.1M glycine, pH 8.0, for 4 hours at room temperature to block unreacted sites. The gel is then washed for five cycles with alternating 50ml volumes of 0.5M NaCl in 0.1M sodium acetate, pH 4.0, and 0.5M NaCl in 0.1M sodium bicarbonate, pH 6.5, to remove non-covalently bound ligand. The gel is loaded into a column (1.5 x 11.2 cm) and equilibrated in 20mM potassium phosphate, pH 6.8.

The combined fractions from the DE-52 column are applied to the column, which is subsequently washed with one column volume (20ml) of the equilibration buffer, and then with 2.5 column volumes (50ml) of 300mM potassium phosphate, pH 6.8. Fractions are assayed for protein using the BCA Protein Assay Reagent (Pierce, Rockford, IL) to make sure that all

extraneous protein has been eluted. Active  $\Delta$ -9 desaturase is eluted from the column with 600mM potassium phosphate, pH 6.8. Active fractions are analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE) on

5 0.75mm thick 8 x 12 cm mini-gels according to the method of Laemmli (*Nature* (1970) 227:680). The running gel contains 10% acrylamide in a 30/0.8 ratio of acrylamide to cross-linker bis-acrylamide. Those fractions containing a predominant band at approximately 43 kD are pooled and stored

10 frozen at  $-70^{\circ}\text{C}$  until final purification. The yield from 50g of seed tissue is ~~25~~ approximately 60 $\mu\text{g}$  of protein as measured by amino acid analysis.

CB

Further purification as described in Example 2 or Example 3 is then applied to the fractions pooled from the

15 ACP-Sepharose column separation.

### Example 2

In this example, a protocol for the final purification of *C. tinctorius* desaturase is described. Seeds are treated

20 in accordance with Example 1.

*Reverse-Phase HPLC:* Fractions from the ACP-Sepharose column are pooled and applied to a Vydac C4 reverse-phase column (0.45 x 15 cm) equilibrated in 0.1% TFA, 7% acetonitrile. After a 10 min wash with 0.1% TFA, the column

25 is eluted with a gradient of increasing acetonitrile (7%-70% v/v) in 0.1% TFA over a period of 45 min. The flow rate is 0.5ml/min throughout. Eluting components are monitored by

absorbance at 214 nm.  $\Delta$ -9 desaturase elutes at about 42 min. (approximately 50% acetonitrile); the major contaminant protein remaining from ACP-affinity chromatography elutes at about 28 min. (approximately 30% acetonitrile). The substantially homogeneous desaturase, which is no longer active, is identified by SDS-PAGE, in which it exhibits a single band corresponding to a molecular weight of approximately 43 kD. The quantity of desaturase protein in the sample may be determined by amino acid analysis.

### Example 3

In this example, a protocol for the final purification of *C. tinctorius* desaturase is described. Seeds are treated in accordance with Example 1.

*Reduction and Alkylation:* Protein is precipitated out of the pooled fraction solutions recovered from the ACP-Sepharose column with 10% (w/v) trichloroacetic acid, washed with cold (-20°C) acetone, and resuspended in 1 ml 500mM Tris-HCl, pH 8.6, containing 6M guanidine-HCl, 10mM EDTA, and 3.2 mM dithiothreitol. After 2 hours, 3.52  $\mu$ mol [ $^3$ H]-iodoacetic acid (64 $\mu$ Ci/ $\mu$ mol, New England Nuclear) is added, and the reaction is allowed to proceed at room temperature in the dark for 2 hours, at which time the reaction is terminated by addition of 1 $\mu$ l (15 $\mu$ mol)  $\beta$ -mercaptoethanol. The sample is then re-precipitated with 10% (w/v) trichloroacetic acid, and the pellet again washed with cold

(-20°C) acetone and resuspended in Laemmli's SDS-sample buffer (*Nature* (1970) 227:680).

*SDS-Polyacrylamide Gel Electrophoresis:* The resulting sample is boiled for 5 min. and then applied to a 1.5 mm thick, 8 x 12 cm, SDS-polyacrylamide mini-gel prepared as described by Laemmli, *supra*. The running gel contains 17.5% acrylamide in a 30:0.13 ratio of acrylamide to cross-linking bis-acrylamide. Separation is achieved by electrophoresis at 15 mA, for 2 hours at 4°C.

*Blotting from SDS-gels to PVDF Membrane:* Proteins are recovered from the gel by electroblotting at 5 mA/cm<sup>2</sup> to a four-layer sandwich of polyvinylidenedifluoride (PVDF) membrane for 2 h at 4°C in a buffer containing 10mM CAPS ("3-[cyclohexylamino]-1-propane-sulfonic acid"), pH 11. The membranes must be wetted in 50% methanol, prior to exposure to the blotting buffer. After blotting, the membrane layers are stained for 1-2 min. in 0.02% Coomassie Blue in 50% methanol, and then destained in 50% methanol. The desaturase is identified as a band corresponding to a molecular weight of about 43 kD; the major contaminant runs at or near the dye front of the gel corresponding to a molecular weight less than 20 kD.

The desaturase band on the PVDF membrane may be applied directly to the Edman sequencer (Applied Biosystems Model 477A) for determination of the N-terminal sequence of the intact protein, or for more extensive sequence determination, may be eluted from the membrane in 40% acetonitrile to

recover pure desaturase in solution. Acetonitrile is removed from the eluted desaturase by evaporation on a Speed-Vac (Savant; Farmingdale, NY), and the substantially homogeneous  $\Delta$ -9 desaturase is resuspended in an appropriate buffer for subsequent proteolytic digestion as described in Example 4. The quantity of desaturase protein present may be determined by amino acid analysis.

Alternatively, if the sample is to be digested with trypsin or gluC protease to generate peptides for amino acid sequence analysis, proteins may be electroblotted to nitrocellulose membranes and stained with Ponceau or amido black.

#### Example 4

In this example, a method for the determination of the amino acid sequence of a desaturase is described.

*Reduction and Alkylation:* Substantially homogenous stearyl-ACP desaturase (See, Example 2) is reduced and alkylated with [ $^3\text{H}$ ]-~~iodoacetic~~ <sup>iodoacetic</sup> acid (See, Example 3), except that the final acetone-washed pellet is resuspended in the appropriate buffer for subsequent proteolysis. Reduction and alkylation assures complete denaturation of the protein so that complete proteolysis can occur. The sample may be alkylated with radiolabeled iodoacetamide or with 4-vinylpyridine instead of [ $^3\text{H}$ ]-~~iodoacetic~~ <sup>iodoacetic</sup> acid in substantially the same manner. Use of iodoacetic acid affords an alkylated

sample with greater solubility, which is advantageous in subsequent sample manipulation.

*Proteolysis:* Substantially pure alkylated samples are digested with endoproteinase lysC. The sample is resuspended  
5 in 100  $\mu$ l of 25 mM Tris-HCl, pH 8.8, containing 1 mM EDTA. Endoproteinase lysC is added to the sample in a protease/desaturase ratio of 1/50 (w/w). Digestion is allowed to proceed at room temperature for 8 hours, at which time another equal amount of protease is added. After 18  
10 more hours, 1  $\mu$ l of concentrated HCl is added to stop proteolysis, and the sample is applied directly to a Vydac C18 reverse-phase column (0.2 x 15 cm) equilibrated in 7% acetonitrile (v/v) in 0.1 mM sodium phosphate, pH 2.2. After washing for 20 min with the equilibration buffer, peptides  
15 are eluted with a gradient in acetonitrile (7-70%, v/v) over 120 min. Flow rate is 50  $\mu$ l/min, throughout. Eluting components are monitored by absorbance at 214 nm, and individual peptide peaks are collected as separate fractions. The peptide fractions are further purified by application to  
20 a second Vydac C18 reverse-phase column (0.2 x 15 cm) equilibrated in 7% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid. Again, after a 20 min wash with equilibration buffer, the substantially pure peptides are eluted with a gradient (7-70%, v/v) of acetonitrile in 0.1%  
25 trifluoroacetic acid over 120 min. The flow rate is 50  $\mu$ l/min, throughout. Eluting components are monitored by absorbance at 214 nm, and individual peptide peaks are

collected as separate fractions. These substantially pure peptides are applied directly to the Edman sequencer (Applied Biosystems, Model 477A) for amino acid sequence analysis.

Alternatively, peptide fraction from the first HPLC

5 purification in phosphate buffer, or from a single chromatography step in trifluoroacetic acid buffer, may be applied directly to the sequencer, but these fractions, in many cases, are not substantially pure and yield mixed or ambiguous sequence information.

10 Other proteases may be used to digest desaturase, including but not limited to trypsin, gluC, and aspN. While the individual digest buffer conditions may be different, the protocols for digestion, peptide separation, purification, and sequencing are substantially the same as those outlined  
15 for the digestion with lysC. Alternatively, desaturase may be digested chemically using cyanogen bromide (Gross *Methods Enzymol* (1967) 11:238-255 or Gross and Witkop *J. Am. Chem. Soc.* (1961) 83:1510), hydroxylamine (Bornstein and Balian *Methods Enzymol.* (1977) 47:132-745), iodosobenzoic acid  
20 (Inglis *Methods Enzymol.* (1983) 91:324-332), or mild acid (Fontana *et al.*, *Methods Enzymol.* (1983) 91:311-317), as described in the respective references.

Fragments generated from these digestion steps of *C. tinctorius* desaturase are presented in Fig. 1 and as SEQ ID

25 NOS: 1-11.



**Example 5**

In this example, the preparation of a plant embryo cDNA bank, using the methods as described in Alexander, et al.

(*Methods in Enzymology* (1987) 154:41-64) and the screening of  
 5 the bank to obtain a desaturase cDNA clone is described.

*C. tinctorius*: A plant embryo cDNA library may be constructed from poly(A)+ RNA isolated from *C. tinctorius* embryos collected at 14-17 days post-anthesis. Poly(A)+ RNA is isolated from polyribosomes by a method initially  
 10 described by Jackson and Larkins (*Plant Physiol.* (1976) 57:5-10) as modified by Goldberg et al. (*Developmental Biol.* (1981) 83:201-217).

The plasmid cloning vector pCGN1703, derived from the commercial cloning vector Bluescribe M13- (Stratagene Cloning  
 15 Systems; San Diego, CA), is made as follows. The polylinker of Bluescribe M13- is altered by digestion with *Bam*HI, treatment with mung bean endonuclease, and blunt-end ligation to create a *Bam*HI-deleted plasmid, pCGN1700. pCGN1700 is digested with *Eco*RI and *Sst*I (adjacent restriction sites) and  
 20 annealed with synthetic complementary oligonucleotides having the sequences

5' CGGATCCACTGCAGTCTAGAGGGCCCGGA 3' (SEQ ID NO: 30) and

5' AATTTCCTGGGCCCCTCTAGACTGCAGTGGATCCGAGCT 3' (SEQ ID NO: 31).

These sequences are inserted to eliminate the *Eco*RI site,  
 25 move the *Bam*HI site onto the opposite side of the *Sst*I (also, sometimes referred to as "SacI" herein) site found in Bluescribe, and to include new restriction sites *Pst*I, *Xba*I,

*ApaI*, *SmaI*. The resulting plasmid pCGN1702, is digested with *HindIII* and blunt-ended with Klenow enzyme; the linear DNA is partially digested with *PvuII* and ligated with T4 DNA ligase in dilute solution. A transformant having the *lac* promoter region deleted is selected (pCGN1703) and is used as the plasmid cloning vector.

Briefly, the cloning method for cDNA synthesis is as follows. The plasmid cloning vector is digested with *SstI* and homopolymer T-tails are generated on the resulting 3'-overhang sticky-ends using terminal deoxynucleotidyl transferase. The tailed plasmid is separated from undigested or un-tailed plasmid by oligo(dA)-cellulose chromatography. The resultant vector serves as the primer for synthesis of cDNA first strands covalently attached to either end of the vector plasmid. The cDNA-mRNA-vector complexes are treated with terminal transferase in the presence of deoxyguanosine triphosphate, generating G-tails at the ends of the cDNA strands. The extra cDNA-mRNA complex, adjacent to the *BamHI* site, is removed by *BamHI* digestion, leaving a cDNA-mRNA-vector complex with a *BamHI* sticky-end at one end and a G-tail at the other. This complex is cyclized using the annealed synthetic cyclizing linker,

5'-GATCCGCGGCCGCGAATTCGAGCTCCCCCCCCC-3' and

3'-GCGCCGGCGCTTAAGCTCGA-5'

which has a *BamHI* sticky-end and a C-tail end. Following ligation and repair the circular complexes are transformed into *E. coli* strain DH5 $\alpha$  (BRL; Gaithersburg, MD) to generate

the cDNA library. The *C. tinctorius* embryo cDNA bank contains between  $3 \times 10^6$  and  $5 \times 10^6$  clones with an average cDNA insert size of approximately 1000 base pairs.

*Probe production Including PCR Reactions:* Two regions  
5 of amino acid sequence (Example 4) with low codon degeneracy are chosen from opposite ends of peptide sequence "Fragment F2" (SEQ ID NO:2) for production of a probe for the plant desaturase cDNA. Two sets of mixed oligonucleotides are designed and synthesized for use as forward (SEQ ID NOS: 21-  
10 24) and reverse (SEQ ID NOS: 25-26) primers, respectively, in the polymerase chain reaction (Saiki et al., *Science* (1985) 230:1350-1354; Oste, *Biotechniques* (1988) 6:162-167). See, Fig. 6. All oligonucleotides are synthesized on an Applied Biosystems 380A DNA synthesizer.

15 Probes to *C. tinctorius* desaturase may be prepared using the peptide sequence "Fragment 2" (SEQ ID NO: 2) identified in Fig. 1. Four types of forward primers were synthesized and labeled 13-1, 13-2, 13-3, and 13-4 (SEQ ID NOS: 21-24, respectively). Two groups of reverse primers were  
20 synthesized and designated 13-5A and 13-6A (SEQ ID NOS: 25-26, respectively). The primer sequences are shown in Fig. 6. These oligonucleotide groups have a redundancy of 64 or less and contain either 20 or 17 bases of coding sequence along with flanking restriction site sequences for *HindIII* or  
25 *EcoRI*. Based on the intervening amino acid sequence between the primer regions on peptide "Fragment 2" (SEQ ID NO: 2) the PCR product is expected to contain 107 base pairs.

Polymerase chain reaction is performed using the cDNA library DNA as template and the possible eight combinations of the four forward and two reverse oligonucleotides as primers in a Perkin-Elmer/Cetus DNA Thermal Cycler (Norwalk, CT) thermocycle file 1 min. 94°C, 2 min. 42°C, 2 min rise from 42°-72°C for 30 cycles, followed by the step cycle file without step rises, 1 min. 94°C, 2 min. 42°C, 3 min. 72°C with increasing 15 sec extensions of the 72°C step for 10 cycles, and a final 10 min. 72°C extension.

10 The product of the 13-4 forward primer (SEQ ID NO: 24) and the 13-5A reverse primer (SEQ ID NO: 25) reaction was ethanol precipitated and then digested with *HindIII* and *EcoRI*, the resulting fragment was subcloned into pUC8 (Vieira and Messing, *Gene* (1982) 19:259-268). Miniprep preparation DNA  
15 (Maniatis et al., *Molecular Cloning: A Laboratory Manual* (1982) Cold Harbor Laboratory, New York) of one clone was sequenced by Sanger dideoxy sequencing (Sanger et al., *Proc. Nat. Acad. Sci. USA* (1977) 74:5463-5467) using the M13 universal and reverse primers. Translation of the resulting  
20 DNA sequence results in a peptide sequence that exactly matches the amino acid sequence in peptide "Fragment F2" (SEQ ID NO: 2).

An exact 50 base oligonucleotide designated DESAT-50 is synthesized to match the sequence of the PCR reaction product  
25 from the first valine residue to the last tyrosine residue.

The probe DSAT-50 5' -  
GTAAGTAGGTAGGGCTTCCTCTGTAATCATATCTCCAACCAAAACAACAA -3' (SEQ

ID NO: 32) is used to probe the *C. tinctorius* embryo cDNA library.

#### *Library screen*

5       The *C. tinctorius* embryo cDNA bank is moved into the cloning vector lambda gt10 (Stratagene Cloning Systems) by digestion of total cDNA with *EcoRI* and ligation to lambda gt10 DNA digested with *EcoRI*. The titer of the resulting library was  $\sim 5 \times 10^5$ /ml. The library is then plated on *E. coli* 10 strain C600 (Huynh, et al., *DNA Cloning Vol. 1* Eds. Glover D.M. IRL Press Limited: Oxford England, pp. 56, 110) at a density of 5000 plaques/150 mm NZY ("NZYM" as defined in Maniatis et al. supra) agar plate to provide over 45,000 plaques for screening. Duplicate lifts are taken of the 15 plaques using NEN Colony Plaque Screen filters by laying precut filters over the plates for  $\sim 1$  minute and then peeling them off. The phage DNA is immobilized by floating the filters on denaturing solution (1.5M NaCl, .05M NaOH) for 1 min., transferring the filters to neutralizing solution (1.5M 20 NaCl, 0.5M Tris-HCl pH 8.0) for 2 min. and then to 2XSSC (1xSSC = 0.15M NaCl; 0.015M Na citrate) for 3 min., followed by air drying. The filters are hybridized with  $^{32}\text{P}$  end-labeled DSAT-50 oligonucleotide (SEQ ID NO: 32) (BRL 5' DNA Terminus Labeling System) by the method of Devlin et al., 25 (*DNA* (1988) 7:499-807) at 42° C overnight, and washed for 30 min. at 50°C in 2XSSC, 0.5% SDS and then twice for 20 min. each at 50°C in 0.1XSSC, 0.5% SDS. Filters are exposed to X-

ray film at  $-70^{\circ}\text{C}$  with a Dupont Cronex intensifying screen for 48 hours.

Clones are detected by hybridization with the DSAT-50 oligonucleotide and plaque purified. The complete nucleotide  
 5 sequence (SEQ ID NO: 12) of the cDNA insert of a clone, pCGN2754, and a partial restriction map thereof are presented in Figures 2 and 7A, respectively. The cDNA insert includes 1533 bases plus a poly(A) track at the 3' end of 100-200 bases. The open reading frame for the desaturase begins at  
 10 the first ATG (nucleotide 106) from the 5' end and stops at nucleotide 1294. The translated amino acid sequence is presented in Fig. 2 and SEQ ID NO: 13. The open reading frame includes a 33 amino acid transit peptide not found in the amino acid sequence of the mature protein. The N-  
 15 terminus of the protein begins at the alanine immediately following the *NcoI* site (nucleotide 202) indicating the site of the transit peptide processing.

### **Example 6**

20 In this example, expression of a plant desaturase in a prokaryote is described.

#### *Desaturase expression construct in E. coli*

A plasmid for expression of desaturase activity in *E. coli* is constructed as follows. The desaturase cDNA clone  
 25 pCGN2754 is digested with *HindIII* and *SalI* and ligated to pCGN2016 (a chloramphenicol resistant version of Bluescript

KS-) digested with *Hind*III and *Xho*I. The resulting plasmid is pCGN1894.

pCGN2016 is prepared by digesting pCGN565 with *Hha*I, and the fragment containing the chloramphenicol resistance gene  
5 is excised, blunted by use of mung bean nuclease, and inserted into the *Eco*RV site of Bluescript KS- (Stratagene: La Jolla, CA) to create pCGN2008. The chloramphenicol resistance gene of pCGN2008 is removed by *Eco*RI/*Hind*III digestion. After treatment with Klenow enzyme to blunt the  
10 ends, the fragment is ligated to *Dra*I digested Bluescript KS-. A clone that has the *Dra*I fragment containing ampicillin resistance replaced with the chloramphenicol resistance is chosen and named pCGN2016.

pCGN565 is a cloning vector based on pUC12-cm (K. Buckley  
15 Ph.D. Thesis, Regulation and expression of the phi X174 lysis gene, University of California, San Diego, 1985), but contains pUC18 linkers (Yanisch-Perron, et al., *Gene* (1985) 53:103-119).

The fragment containing the mature coding region of the  $\Delta$ -9 desaturase, 3'-noncoding sequences and poly(A) tails is  
20 subcloned from pCGN1894 digested with *Nco*I and *Asp*718 into pUC120, an *E. coli* expression vector based on pUC118 (Vieira and Messing, *Methods in Enzymology* (1987) 153:3-11) with the lac region inserted in the opposite orientation and an *Nco*I site at the ATG of the lac peptide (Vieira, J. Ph.D. Thesis,  
25 University of Minnesota, 1988). The *E. coli* desaturase expression plasmid is designated pCGN3201. The desaturase

sequences are inserted such that they are aligned with the lac transcription and translation signals.

*Expression of Desaturase in E.coli*

5        Single colonies of *E. coli* strain 7118 (Maniatis *et al.*, *supra*) containing pUC120 or pCGN3201 are cultured in 80 mls each of ECLB broth, 300 mg/L penicillin. The cells are induced by the addition of 1mM IPTG. Cells are grown overnight (18 hrs) at 37° C.

10       Eighty mls of overnight cultures of *E. coli* (induced and uninduced) containing pUC120 or pCGN3201 are centrifuged at 14,800 x g for 15 min. The pelleted cells are resuspended in 3 mls 20 mM phosphate buffer, pH 6.8. Resuspended cells were broken in a french press at 16,000 psi. Broken cell mixtures  
15 are centrifuged 5000xg for 5 min. 100 µl of each supernatant is applied to a G-25 Sephadex gel filtration centrifugal column (Boehringer Mannheim Biochemicals), equilibrated in 20mM phosphate buffer pH 6.8. Columns are spun for 4 min at 5000xg. Effluent was collected and used as enzyme source in  
20 the desaturase assay.

Desaturase activity is assayed as described in Example 1. Both pUC120-containing, IPTG-induced cells and uninduced cells do not express detectable stearyl-ACP desaturase activity. The pCGN3201 IPTG-induced extract contains 8.22  
25 nmol/min of desaturase activity. pCGN3201 uninduced extracts contains 6.45 nmol/min of activity. The pCGN3201 IPTG-induced



extract shows 21.5% more activity than the uninduced pCGN3201 extract.

*Detection of induced protein in E. coli.*

5        Extracts of overnight cultures of *E. coli* strain 7118  
(Maniatis et al. supra) containing pCGN3201 or pUC120 grown  
in ECLB containing 300 mg/L penicillin induced with 1mM IPTG  
are prepared as follows. 1.5 ml of overnight culture grown  
shaking at 37°C are pelleted in Eppendorf tubes for 10 min at  
10 10-13,000 µg. Pellets are resuspended in 150 µl SDS sample  
buffer (0.05M Tris-HCl, pH6.8, 1% SDS, 5% β-mercaptoethanol,  
10% glycerol and 0.005% bromophenol blue) and boiled for 10  
min. 25 µl of each sample are electrophoresed on a 10%  
polyacrylamide gel (Laemmli, Nature (1970) 227:680) at 25 mA  
15 for 5 hours. Gels are stained in 0.05% Coomassie Brilliant  
Blue, 25% isopropanol and 10% acetic acid and destained in  
10% acetic acid and 10% isopropanol. A band is detected at a  
position just below the 43,000 MW protein marker (SDS PAGE  
standard, Low molecular weight, BioRad, Richmond CA) in the  
20 pCGN3201 extracts that is not present in the pUC120 extracts.  
This is the approximate molecular weight of mature desaturase  
protein.

*Requirement for Spinach Ferredoxin*

25        Stearoyl-ACP desaturase can also be expressed in *E. coli*  
by subcloning into the *E. coli* expression vector pET8c  
(Studier, et al., Methods Enzymol. (1990) 185:60-89). The

mature coding region (plus an extra Met codon) of the desaturase cDNA with accompanying 3'-sequences is inserted as an *Nco*I - *Sma*I fragment into pET8c at the *Nco*I and *Bam*HI sites (after treatment of the *Bam*HI site with Klenow fragment of DNA polymerase to create a blunt end) to create pCGN3208. The plasmid pCGN3208 is maintained in *E. coli* strain BL21(DE3) which contains the T7 polymerase gene under the control of the isopropyl-b-D-thiogalactopyranoside (IPTG)-inducible *lacUV5* promoter (Studier et al., supra).

10 *E. coli* cells containing pCGN3208 are grown at 37°C to an OD<sub>595</sub> of ~0.7 in NZY broth containing 0.4% (w/v) glucose and 300 mg/liter penicillin, and then induced for 3 hours with 0.4 mM IPTG. Cells are pelleted from 1 ml of culture, dissolved in 125 µl of SDS sample buffer (10) and heated to 15 100°C for 10 min. Samples are analyzed by SDS polyacrylamide gel electrophoresis at 25 mA for 5 h. Gels are stained in 0.05% Coomassie Brilliant Blue, 25% (v/v) isopropanol and 10% (v/v) acetic acid. A band is detected at a position just below the 43,000 MW protein marker (SDS PAGE standard, Low 20 Molecular Weight, BioRad, Richmond, CA) in the pCGN3208 extract that is not present in the pET8c extracts. This is the approximate molecular weight of mature desaturase protein.

For activity assays, cells are treated as described 25 above and extracts are used as enzyme source in the stearoyl-ACP desaturase assay as described in Example 1. The extract from IPTG-induced pCGN3208 cells contains 8.61 nmol/min/mg

protein of desaturase activity. The extract from pCGN3208 uninduced cells contains 1.41 nmol/min/mg protein of desaturase activity. The extract from pCGN3208 induced cells, thus has approximately 6-fold greater activity than the extract from uninduced pCGN3208 cells. Extracts from both induced and uninduced cells of pET8c do not contain detectable stearyl-ACP desaturase activity.

Samples are also assayed as described in Example 1, but without the addition of spinach ferredoxin, to determine if the *E. coli* ferredoxin is an efficient electron donor for the desaturase reaction. Minimal activity is detected in *E. coli* extracts unless spinach ferredoxin is added exogenously.

#### Example 7

In this example, the preparation of an ACP expression cassette containing a plant desaturase in a binary vector suitable for plant transformation is described.

#### *ACP Expression Cassette*

An expression cassette utilizing 5'-upstream sequences and 3'-downstream sequences obtainable from *B. campestris* ACP gene can be constructed as follows.

A 1.45kb *Xho*I fragment of Bcg 4-4 (Fig. 9 and SEQ ID NO: 28) containing 5'-upstream sequences is subcloned into the cloning/sequencing vector Bluescript+ (Stratagene Cloning Systems, San Diego, CA). The resulting construct, pCGN1941, is digested with *Xho*I and ligated to a chloramphenicol

resistant Bluescript M13+ vector, pCGN2015 digested with *Xho*I. pCGN2015 is prepared as described for pCGN2016 (See, Example 6) except that the *Eco*RI/*Hind*III "chloramphenicol" fragment isolated from pCGN2008 is ligated with the 2273 bp fragment of Bluescript KS<sup>+</sup> (Stratagene; LaJolla, CA) isolated after digestion with *Dra*I. This alters the antibiotic resistance of the plasmid from penicillin resistance to chloramphenicol resistance. The chloramphenicol resistant plasmid is pCGN1953.

3'-sequences of Bcg 4-4 are contained on an *Sst*I/*Bgl*II fragment cloned in the *Sst*I/*Bam*HI sites of M13 Bluescript+ vector. This plasmid is named pCGN1940. pCGN1940 is modified by *in vitro* site-directed mutagenesis (Adelman et al., DNA (1983) 2:183-193) using the synthetic oligonucleotide 5'-CTTAAGAAGTAACCCGGGCTGCAGTTTTAGTATTAAGAG-3' (SEQ ID NO: 33) to insert *Sma*I and *Pst*I restriction sites immediately following the stop codon of the reading frame for the ACP gene 18 nucleotides from the *Sst*I site. The 3'-noncoding sequences from this modified plasmid, pCGN1950, are moved as a *Pst*I-*Sma*I fragment into pCGN1953 cut with *Pst*I and *Sma*I. The resulting plasmid pCGN1977 comprises the ACP expression cassette with the unique restriction sites *Eco*RV, *Eco*RI and *Pst*I available between the 1.45kb 5' and 1.5 kb of 3'-noncoding sequences (SEQ ID NO: 28) for the cloning of genes to be expressed under regulation of these ACP gene regions.

### *Desaturase Expression in Plants*

Desaturase cDNA sequences from pCGN2754 are inserted in the ACP expression cassette, pCGN1977, as follows. pCGN2754 is digested with *HindIII* (located 160 nucleotides upstream of the start codon) and *Asp718* located in the polylinker outside the poly(A) tails. The fragment containing the coding region for desaturase was blunt-ended using DNA polymerase I and ligated to pCGN1977 digested with *EcoRV*. A clone containing the desaturase sequences in the sense orientation with respect to the ACP promoter is selected and called pCGN1895. This expression cassette may be inserted into a binary vector, for example, for *Agrobacterium*-mediated transformation, or employed in other plant transformation techniques.

15

### *Binary Vector and Agrobacterium Transformation*

The fragment containing the pCGN1895 expression sequences ACP 5'/desaturase/ACP 3' is cloned into a binary vector pCGN1557 (described below) for *Agrobacterium* transformation by digestion with *Asp718* and *XbaI* and ligation to pCGN1557 digested with *Asp718* and *XbaI*. The resulting binary vector is called pCGN1898.

pCGN1898 is transformed into *Agrobacterium tumefaciens* strain EHA101 (Hood, et al., *J. Bacteriol.* (1986) 168:1291-1301) as per the method of Holsters, et al., *Mol. Gen. Genet.* (1978) 163:181-187.

RNA blot analysis of seeds (T2) from T1 plants show the presence of a mRNA species for the inserted *C. tinctorius* desaturase, but the amount of message is low compared to endogenous levels of mRNA for the *Brassica* desaturase, suggesting that the expression levels were insufficient to significantly increase the amount of desaturase enzyme above that normally present. This is consistent with the negative results from oil, desaturase activity and Western blot analyses.

10

#### Construction of pCGN1557

pCGN1557 (McBride and Summerfelt, *Plant Molecular Biology* (1990) 14(2):269-276) is a binary plant transformation vector containing the left and right T-DNA borders of *Agrobacterium tumefaciens* octopine Ti-plasmid pTiA6 (Currier and Nester, *supra*, the gentamycin resistance gene of pPH1JI (Hirsch and Beringer, *supra*), an *Agrobacterium rhizogenes* Ri plasmid origin of replication from pLJbB11 (Jouanin et al., *supra*), a 35S promoter-kanR-tml3' region capable of conferring kanamycin resistance to transformed plants, a ColE1 origin of replication from pBR322 (Bolivar et al., *supra*), and a *lacZ'* screenable marker gene from pUC18 (Yanish-Perron et al., *supra*).

There are three major intermediate constructs used to generate pCGN1557:

pCGN1532 (see below) contains the pCGN1557 backbone, the pRi plasmid origin of replication, and the ColE1 origin of replication.

pCGN1546 (see below) contains the CaMV35S5'-kan<sup>R</sup>-tml3' plant selectable marker region.

pCGN1541b (see below) contains the right and left T-DNA borders of the *A. tumefaciens* octopine Ti-plasmid and the *lacZ'* region from pUC19.

To construct pCGN1557 from the above plasmids, pCGN1546 is digested with *Xho*I, and the fragment containing the CaMV 35S5'-kan<sup>R</sup>-tml3' region is cloned into the *Xho*I site of pCGN1541b to give the plasmid pCGN1553, which contains T-DNA/left border/CaMV 35S5'-kan<sup>R</sup>-tml3'/*lacZ'*/T-DNA left border. pCGN1553 is digested with *Bgl*III, and the fragment containing the T-DNA/left border/CaMV35S5'-kan<sup>R</sup>-tml3'/*lacZ'*/T-DNA left border region is ligated into *Bam*HI-digested pCGN1532 to give the complete binary vector, pCGN1557.

#### pCGN1532

The 3.5kb *Eco*RI-*Pst*I fragment containing the gentamycin resistance gene is removed from pPh1JI (Hirsch and Beringer, *Plasmid* (1984) 12:139-141) by *Eco*RI-*Pst*I digestion and cloned into *Eco*RI-*Pst*I digested pUC9 (Vieira and Messing, *Gene* (1982) 19:259-268) to generate pCGN549. *Hind*III-*Pst*I digestion of pCGN549 yields a 3.1 kb fragment bearing the gentamycin resistance gene, which is made blunt ended by the Klenow fragment of DNA polymerase I and cloned into *Pvu*II

digested pBR322 (Bolivar et al., *Gene* (1977) 2:95-113) to create pBR322Gm. pBR322Gm is digested with *Dra*I and *Sph*I, treated with Klenow enzyme to create blunt ends, and the 2.8 kb fragment cloned into the *Ri* origin-containing plasmid

5 pLJbB11 (Jouanin et al., *Mol. Gen. Genet.* (1985) 201:370-374) which has been digested with *Apa*I and made blunt-ended with Klenow enzyme, creating pLHbB11Gm. The extra *ColE1* origin and the kanamycin resistance gene are deleted from pLHbB11Gm by digestion with *Bam*HI followed by self closure to create  
10 pGmB11. The *Hind*II site of pGmB11 is deleted by *Hind*III digestion followed by treatment with Klenow enzyme and self closure, creating pGmB11-H. The *Pst*I site of pGmB11-H is deleted by *Pst*I digestion followed by treatment with Klenow enzyme and self closure, creating pCGN1532.

15

#### Construction of pCGN1546

The 35S promoter-tml3' expression cassette, pCGN986, contains a cauliflower mosaic virus 35S (CaMV35) promoter and a T-DNA tml 3'-region with multiple restriction sites between  
20 them. pCGN986 is derived from another cassette, pCGN206, containing a CaMV35S promoter and a different 3' region, the CaMV region VI 3'-end. The CaMV 35S promoter is cloned as an *Alu*I fragment (bp 7144-7734) (Gardner et. al., *Nucl. Acids Res.* (1981) 9:2871-2888) into the *Hinc*II site of M13mp7  
25 (Messing, et. al., *Nucl. Acids Res.* (1981) 9:309-321) to create C614. An *Eco*RI digest of C614 produced the *Eco*RI fragment from C614 containing the 35S promoter which is



cloned into the *EcoRI* site of pUC8 (Vieira and Messing, *Gene* (1982) 19:259) to produce pCGN147.

pCGN148a containing a promoter region, selectable marker (KAN with 2 ATG's) and 3' region, is prepared by digesting  
5 pCGN528 with *Bgl*III and inserting the *Bam*HI-*Bgl*III promoter fragment from pCGN147. This fragment is cloned into the *Bgl*III site of pCGN528 so that the *Bgl*III site is proximal to the kanamycin gene of pCGN528.

The shuttle vector used for this construct pCGN528, is  
10 made as follows: pCGN525 is made by digesting a plasmid containing Tn5 which harbors a kanamycin gene (Jorgenson et. al., *Mol. Gen. Genet.* (1979) 177:65) with *Hind*III-*Bam*HI and inserting the *Hind*III-*Bam*HI fragment containing the kanamycin gene into the *Hind*III-*Bam*HI sites in the tetracycline gene of  
15 pACYC184 (Chang and Cohen, *J. Bacteriol.* (1978) 134:1141-1156). pCGN526 was made by inserting the *Bam*HI fragment 19 of pTiA6 (Thomashow et. al., *Cell* (1980) 19:729-739), modified with *Xho*I linkers inserted into the *Sma*I site, into the *Bam*HI site of pCGN525. pCGN528 is obtained by deleting  
20 the small *Xho*I fragment from pCGN526 by digesting with *Xho*I and religating.

pCGN149a is made by cloning the *Bam*HI-kanamycin gene fragment from pMB9KanXXI into the *Bam*HI site of pCGN148a. pMB9KanXXI is a pUC4K variant (Vieira and Messing, *Gene*  
25 (1982) 19:259-268) which has the *Xho*I site missing, but contains a functional kanamycin gene from Tn903 to allow for efficient selection in *Agrobacterium*.

pCGN149a is digested with *Hind*III and *Bam*HI and ligated to pUC8 digested with *Hind*III and *Bam*HI to produce pCGN169. This removes the Tn903 kanamycin marker. pCGN565 (see pCGN2016 description) and pCGN169 are both digested with  
5 *Hind*III and *Pst*I and ligated to form pCGN203, a plasmid containing the CaMV 35S promoter and part of the 5'-end of the Tn5 kanamycin gene (up to the *Pst*I site, Jorgenson et al., (1979), *supra*). A 3'-regulatory region is added to pCGN203 from pCGN204 (an *Eco*RI fragment of CaMV (bp 408-6105)  
10 containing the region VI 3' cloned into pUC18 (Yanisch-Perron, et al., *Gene* (1985) 33:103-119) by digestion with *Hind*III and *Pst*I and ligation. The resulting cassette, pCGN206, is the basis for the construction of pCGN986.

The pTiA6 T-DNA tml 3'-sequences are subcloned from the  
15 *Bam*19 T-DNA fragment (Thomashow et al., (1980) *supra*) as a *Bam*HI-*Eco*RI fragment (nucleotides 9062 to 12,823, numbering as in Barker et al., *Plant Mol. Biol.* (1982) 2:335-350) and combined with the pACYC184 (Chang and Cohen (1978), *supra*) origin of replication as an *Eco*RI-*Hind*III fragment and a  
20 gentamycin resistance marker (from plasmid pLB41), obtained from D. Figurski) as a *Bam*HI-*Hind*III fragment to produce pCGN417.

The unique *Sma*I site of pCGN417 (nucleotide 11,207 of the *Bam*19 fragment) is changed to a *Sac*I site using linkers  
25 and the *Bam*HI-*Sac*I fragment is subcloned into pCGN565 to give pCGN971. The *Bam*HI site of pCGN971 is changed to an *Eco*RI site using linkers. The resulting *Eco*RI-*Sac*I fragment

containing the tml 3' regulatory sequences is joined to pCGN206 by digestion with *EcoRI* and *SacI* to give pCGN975.

The small part of the Tn5 kanamycin resistance gene is deleted from the 3'-end of the CaMV 35S promoter by digestion with *SalI* and *BglII*, blunting the ends and ligation with *SalI* linkers. The final expression cassette pCGN986 contains the CaMV 35S promoter followed by two *SalI* sites, an *XbaI* site, *BamHI*, *SmaI*, *KpnI* and the tml 3' region (nucleotides 11207-9023 of the T-DNA).

The 35S promoter-tml 3' expression cassette, pCGN986 is digested with *HindIII*. The ends are filled in with Klenow polymerase and *XhoI* linkers added. The resulting plasmid is called pCGN986X. The *BamHI-SacI* fragment of pBRX25 (see below) containing the nitrilase gene is inserted into *BamHI-SacI* digested pCGN986X yielding pBRX66.

Construction of pBRX25 is described in U.S. Letters Patent 4,810,648, which is hereby incorporated by reference. Briefly, the method is as follows: The nucleotide sequence of a 1212-bp *PstI-HincII* DNA segment encoding the bromoxynil-specific nitrilase contains 65-bp of 5' untranslated nucleotides. To facilitate removal of a portion of these excess nucleotides, plasmid pBRX9 is digested with *PstI*, and treated with nuclease *Bal31*. *BamHI* linkers are added to the resulting ends. *BamHI-HincII* fragments containing a functional bromoxynil gene are cloned into the *BamHI-SmaI* sites of pCGN565. The resulting plasmid, pBRX25, contains only 11 bp of 5' untranslated bacterial sequence.

pBRX66 is digested with *Pst*I and *Eco*RI, blunt ends generated by treatment with Klenow polymerase, and *Xho*I linkers added. The resulting plasmid pBRX68 now has a tml 3' region that is approximately 1.1kb. pBRX68 is digested with *Sal*I and *Sac*I, blunt ends generated by treatment with Klenow polymerase and *Eco*RI linkers added. The resulting plasmid, pCGN986XE is a 35S promoter - tml 3' expression cassette lacking the nitrilase gene.

The Tn5 kanamycin resistance gene is then inserted into pCGN986XE. The 1.0 kb *Eco*RI fragment of pCGN1536 (see pCGN1547 description) is ligated into pCGN986XE digested with *Eco*RI. A clone with the Tn5 kanamycin resistance gene in the correct orientation for transcription and translation is chosen and called pCGN1537b. The 35S promoter Kan<sup>R</sup>-tml 3' region is then transferred to a ~~chloramphenicol~~ resistant plasmid backbone. pCGN786, (a pUC-CAM based vector with the synthetic oligonucleotide 5'

GGAATTCGTCGACAGATCTCTGCAGCTCGAGGGATCCAAGCTT 3' (SEQ ID NO:

34) containing the cloning sites *Eco*RI, *Sal*I, *Bgl*III, *Pst*I,

*Xho*I, *Bam*HI, and *Hind*III inserted into pCGN566, pCGN566

contains the *Eco*HI-*Hind*III linker of pUC18 inserted into the *Eco*KI-*Hind*III sites of pUC13-cm (K. Buckler (1985) *supra*)) is digested with *Xho*I and the *Xho*I fragment of pCGN1537b

containing the 35S promoter - Kan<sup>R</sup>-tml 3' region is ligated

in. The resulting clone is termed pCGN1546.

**pCGN1541b**

pCGN565RB $\alpha$ 2X (see below) is digested with *Bgl*III and *Xho*I, and the 728bp fragment containing the T-DNA right border piece and the *lacZ'* gene is ligated with *Bgl*III-*Xho*I digested pCGN65 $\Delta$ KX-S+K (see below), replacing the *Bgl*III-*Xho*I right border fragment of pCGN65 $\Delta$ KX-S+K. The resulting plasmid, pCGN65 $\alpha$ 2X contains both T-DNA borders and the *lacZ'* gene. The *Cla*I fragment of pCGN65 $\alpha$ 2X is replaced with an *Xho*I site by digesting with *Cla*I blunting the ends using the Klenow fragment, and ligating with *Xho*I linker DNA, resulting in plasmid pCGN65 $\alpha$ 2XX. pCGN65 $\alpha$ 2XX is digested with *Bgl*III and *Eco*RV, treated with the Klenow fragment of DNA polymerase I to create blunt ends, and ligated in the presence of *Bgl*III linker DNA, resulting in pCGN65 $\alpha$ 2XX'. pCGN65 $\alpha$ 2XX' is digested with *Bgl*III and ligated with *Bgl*III digested pCGN1538 (see below), resulting in pCGN1541a, which contains both plasmid backbones. pCGN1541a is digested with *Xho*I and religated. Ampicillin resistant, ~~chloramphenicol~~ sensitive clones are chosen, which lack the pACYC184-derived backbone, creating pCGN1541b.

pCGN1538 is generated by digesting pBR322 with *Eco*RI and *Pvu*II, treating with Klenow to generate blunt ends, and ligating with *Bgl*III linkers. pCGN1538 is ampicillin resistant, tetracycline sensitive.

**pCGN65ΔKX-S+K**

pCGN501 is constructed by cloning a 1.85 kb *EcoRI*-*XhoI* fragment of pTiA6 (Currier and Nester, *J. Bact.* (1976) 126:157-165) containing bases 13362-15208 (Barker et al., *Plant Mo. Biol.* (1983) 2:335-350) of the T-DNA (right border), into *EcoRI*-*SalI* digested M13mp9 (Vieira and Messing, *Gene* (1982) 19:259-268). pCGN502 is constructed by cloning a 1.6 kb *HindIII*-*SmaI* fragment of pTiA6, containing bases 602-2212 of the T-DNA (left border), into *HindIII*-*SmaI* digested M13mp9. pCGN501 and pCGN502 are both digested with *EcoRI* and *HindIII* and both T-DNA-containing fragments cloned together into *HindIII* digested pUC9 (Vieira and Messing, *Gene* (1982) 19:259-268) to yield pCGN503, containing both T-DNA border fragments. pCGN503 is digested with *HindIII* and *EcoRI* and the two resulting *HindIII*-*EcoRI* fragments (containing the T-DNA borders) are cloned into *EcoRI* digested pHc79 (Hohn and Collins, *Gene* (1980) 11:291-298) to generate pCGN518. The 1.6kb *KpnI*-*EcoRI* fragment from pCGN518, containing the left T-DNA border, is cloned into *KpnI*-*EcoRI* digested pCGN565 to generate pCGN580. The *BamHII*-*BglIII* fragment of pCGN580 is cloned into the *BamHI* site of pACYC184 (Chang and Cohen, *J. Bacteriol.* (1978) 134:1141-1156) to create pCGN51. The 1.4 kb *BamHI*-*SphI* fragment of pCGN60 containing the T-DNA right border fragment, is cloned into *BamHI*-*SphI* digested pCGN51 to create pCGN65, which contains the right and left T-DNA borders.

pCGN65 is digested with *KpnI* and *XbaI*, treated with Klenow enzyme to create blunt ends, and ligated in the presence of synthetic *BglIII* linker DNA to create pCGN65ΔKX. pCGN65ΔKX is digested with *SalI*, treated with Klenow enzyme  
 5 to create blunt ends, and ligated in the presence of synthetic *XhoI* linker DNA to create pCGN65ΔKX-S+X.

pCGN565RBα2X

pCGN451 (see below) is digested with *HpaI* and ligated in  
 10 the presence of synthetic *SphI* linker DNA to generate pCGN55. The *XhoI-SphI* fragment of pCGN55 (bp13800-15208, including the right border, of *Agrobacterium tumefaciens* T-DNA; (Barker et al., *Gene* (1977) 2:95-113) is cloned into *SalI-SphI* digested pUC19 (Yanisch-Perron et al., *Gene* (1985) 53:103-  
 15 119) to create pCGN60. The 1.4 kb *HindIII-BamHI* fragment of pCGN60 is cloned into *HindIII-BamHI* digested pSP64 (Promega, Inc.) to generate pCGN1039. pCGN1039 is digested with *SmaI* and *NruI* (deleting bp14273-15208; (Barker et al., *Gene* (1977) 2:95-113) and ligated in the presence of synthetic *BglIII*  
 20 linker DNA creating pCGN1039ΔNS. The 0.47 kb *EcoRI-HindIII* fragment of pCGN1039ΔNS is cloned into *EcoRI-HindIII* digested pCGN565 to create pCGN565RB. The *HindIII* site of pCGN565RB is replaced with an *XhoI* site by digesting with *HindIII*, treating with Klenow enzyme, and ligating in the presence of  
 25 synthetic *XhoI* linker DNA to create pCGN565RB-H+X.

pUC18 (Norrandar et al., *Gene* (1983) 26:101-106) is digested with *HaeII* to release the *lacZ'* fragment, treated

with Klenow enzyme to create blunt ends, and the *lacZ'*-containing fragment ligated into pCGN565RB-H+X, which had been digested with *AccI* and *SphI* and treated with Klenow enzyme in such a orientation that the *lacZ'* promoter is proximal to the right border fragment; this construct, pCGN565RBQ2x is positive for *lacZ'* expression when plated on an appropriate host and contains bp 13990-14273 of the right border fragment (Barker et al., *Plant Mo. Biol.* (1983) 2:335-350) having deleted the *AccI-SphI* fragment (bp 13800-13990).

#### 10      pCGN451

pCGN451 contains an *ocs5'*-*ocs3'* cassette, including the T-DNA right border, cloned into a derivative of pUC8 (Vieira and Messing, *supra*). The modified vector is derived by digesting pUC8 with *HincII* and ligating in the presence of synthetic linker DNA, creating pCGN416, and then deleting the *EcoRI* site of pCGN416 by *EcoRI* digestion followed by treatment with Klenow enzyme and self-ligation to create pCGN426.

The *ocs5'*-*ocs3'* cassette is created by a series of steps from DNA derived from the octopine Ti-plasmid pTiA6 (Currier and Nester, *supra*). To generate the 5'end, which includes the T-DNA right border, an *EcoRI* fragment of pTiA6 (bp 13362-16202 (the numbering is by Barker, et al., (*Plant Mol. Bio* (1983) 2:335-350) for the closely related Ti plasmid pTil5955)) is removed from pVK232 (Knauf and Nester, *Plasmid* (1982) 8:45) by *EcoRI* digestion and cloned into *EcoRI*



digested pACYC184 (Chang and Cohen, *supra*) to generate pCGN15.

The 2.4kb *Bam*HI-*Eco*RI fragment (bp 13774-16202) of pCGN15 is cloned into *Eco*RI-*Bam*HI digested pBR322 (Bolivar, *et al.*, *supra*) to yield pCGN429. The 412 bp *Eco*RI-*Bam*HI fragment (bp 13362-13772) of pCGN15 is cloned into *Eco*RI-*Bam*HI digested pBR322 to yield pCGN407. The cut-down promoter fragment is obtained by digesting pCGN407 with *Xmn*I (bp 13512), followed by resection with *Bal*31 exonuclease, ligation of synthetic *Eco*RI linkers, and digestion with *Bam*HI. Resulting fragments of approximately 130 bp are gel purified and cloned into M13mp9 (Vieira and Messing, *supra*) and sequenced. A clone, I-4, in which the *Eco*RI linker has been inserted at bp 1362 between the transcription initiation point and the translation initiation codon is identified by comparison with the sequence of de Greve, *et al.*, (*J. Mol. Appl. Genet.* (1982) 1:499-512). The *Eco*RI cleavage site is at position 13639, downstream from the mRNA start site. The 141 bp *Eco*RI-*Bam*HI fragment of I-4, containing the cut-down promoter, is cloned into *Eco*RI-*Bam*HI digested pBR322 to create pCGN428. The 141 bp *Eco*RI-*Bam*HI promoter piece from pCGN428, and the 2.5 kb *Eco*RI-*Bam*HI *ocs*5' piece from pCGN429 are cloned together into *Eco*RI digested pUC19 (Vieira and Messing, *supra*) to generate pCGN442, reconstructing the *ocs* upstream region with a cut-down promoter section.

To generate the *ocs*3' end, the *Hind*III fragment of pLB41 (D. Figurski, UC San Diego) containing the gentamycin

resistance gene is cloned into *HindIII* digested pACYC184 (Chang and Cohen, *supra*) to create pCGN413b. The 4.7 kb *BamHI* fragment of pTiA6 (*supra*), containing the *ocs3'* region, is cloned into *BamHI* digested pBR325 (F. Bolivar, *Gene* (1978) 4:121-136) to create 33c-19. The *SmaI* site at position 11207 (Barker, *supra*) of 33c-19 is converted to an *XhoI* site using a synthetic *XhoI* linker, generating pCCG401.2. The 3.8 kb *BamHI-EcoRI* fragment of pCGN401.2 is cloned into *BamHI-EcoRI* digested pCGN413b to create pCGN419.

10 The *ocs5'-ocs3'* cassette is generated by cloning the 2.64 kb *EcoRI* fragment of pCGN442, containing the 5' region, into *EcoRI* digested pCGN419 to create pCNG446. The 3.1kb *XhoI* fragment of pCGN446, having the *ocs5'* region (bp 13639-15208) and *ocs3'* region (bp 11207-12823), is cloned into the 15 *XhoI* site of pCGN426 to create pCGN451.

### Example 8

In this example, the preparation of a Bce-4 expression cassette containing a plant desaturase is described.

20 The desaturase cDNA clone from pCGN2754 prepared as described in Example 5, is modified by *in vitro* mutagenesis to insert restriction sites immediately upstream of the ATG start codon and downstream of the TGA stop codon. A single-stranded template DNA is prepared for the mutagenesis reaction from pCGN1894 (described in Example 6) as described by Messing, (*Methods in Enzymol.* (1983) 101:20-79). Synthetic oligonucleotides are synthesized on an Applied

Biosystems 380A DNA synthesizer. The oligonucleotides used are

5'-CCATTTTGTATCTTCCTCGAGCCCGGGCTGCAGTTCTTCTTCTTCTTG-3'

(SEQ ID NO: 35) for the 5'-mutagenesis and

5 5'-GCTCGTTTTTTTTTCTCTGCAGCCCGGGCTCGAGTCACAGCTTCACC -3'

(SEQ ID NO: 36) for the 3'-mutagenesis; both add *Pst*I, *Sma*I and *Xho*I sites flanking the coding region. Both

oligonucleotides are 5'-phosphorylated (BRL 5'-Terminus labelling kit) and used for mutagenesis with the pCGN1894

10 template by the procedure of Adelman et al. (*DNA* (1983) 2:183-193). Alternatively, the desired restriction sites may be inserted by PCR, using the 3' oligo described above (SEQ ID NO: 36) and another oligo,

5' ACTGACTGCAGCCCGGGCTCGAGGAAGATCAAAAATGGCTCTTC 3' (SEQ ID

15 NO: 37) for the 3' and 5' primers, respectively. The template in this polymerase chain reaction is DNA from pCGN1894. The *Xho*I fragment from the resulting clone can be subcloned into the Bce4 expression cassette, pCGN1870 (described below) at the unique *Xho*I site. This

20 Bce4/desaturase expression cassette can then be inserted in a suitable binary vector, transformed into *Agrobacterium tumefaciens* strain EHA101 and used to transform plants as provided in Example 10.

## 25 Bce-4 Expression Cassette

pCGN1870 is a Bce-4 expression cassette containing 5' and 3' regulatory regions of the Bce-4 gene and may be

derived from the Bce-4 sequence found in pCGN1857, which was deposited with the ATCC on March 9, 1990, and assigned accession number 68251, or by methods known to one skilled in the art from the sequence (SEQ ID NO: 27) provided in Fig. 8.

5 The Bce 4 gene may be isolated as follows:

The *Cla*I fragment of pCGN1857, containing the Bce4 gene is ligated into *Cla*I digested Bluescript KS+ (Stratagene; La Jolla, CA), producing pCGN1864. Single stranded DNA is made from pCGN1864 and altered by *in vitro* mutagenesis using the  
10 oligonucleotides

BCE45P:

(5'GAGTAGTGAACCTTCATGGATCCTCGAGGTCTTGAAAACCTAGA3') (SEQ ID NO: 38) and

BCE43P:

15 (5'CAATGTCTTGAGAGATCCCGGGATCCTTAACAACTAGGAAAAGG3') (SEQ ID NO: 39)

as described by Adelman *et al.* (*DNA* (1983) 2:183-193). The oligonucleotide BSCP2 (5'GTAAGACACGACTTATCGCCACTG3') (SEQ ID NO: 40), complementary to a portion of Bluescript, is  
20 included in the reaction to improve the yield of double-stranded DNA molecules. The resulting plasmid, pCGN1866, contains *Xho*I and *Bam*HI sites (from BCE45P) immediately 5' to the Bce4 start codon and *Bam*HI and *Sma*I sites (from BCE43P) immediately 3' to the Bce4 stop codon. The *Cla*I fragment of  
25 pCGN1866, containing the mutagenized sequences, is inserted

into the *Cla*I site of pCGN2016 (described in Example 6), producing pCGN1866C. The *Cla*I fragment of pCGN1866C is used to replace the corresponding wild-type *Cla*I fragment of pCGN1867 (described below) to produce pCGN1868. Bce4 coding sequences are removed by digestion of pCGN1868 with *Bam*HI and recircularization of the plasmid to produce pCGN1870. The Bce4 expression cassette, pCGN1870, contains 7.4 kb of 5' regulatory sequence and 1.9 kb of 3' regulatory sequence derived from the Bce4 genomic clone separated by the cloning sites, *Xho*I, *Bam*HI, and *Sma*I. Desaturase sequences in sense or anti-sense orientation may be inserted into the cassette via the cloning sites and the resulting construct may be employed in a plant transformation technique.

15      pCGN1867

The *Bam*HI and *Sma*I sites of pUC18 are removed by *Bam*HI-*Sma*I digestion and recircularizing of the plasmid, without repair of the ends, to produce pCGN1862. The *Pst*I fragment of pCGN1857, containing the Bce4 gene, is inserted into the *Pst*I site of pCGN1862 to produce pCGN1867.

**Example 9**

In this example, the preparation of a napin 1-2 expression cassette containing a plant desaturase is described.

### *Preparation of Desaturase Clone*

The desaturase cDNA clone from pCGN2754 is prepared and modified as described in Example 8. The *Xho*I fragment from the resulting clone can be subcloned into the napin 1-2

5 expression cassette, pCGN1808 (described below) at the unique *Xho*I site. This napin 1-2/desaturase expression cassette can then be inserted into a suitable binary vector, transformed into *A. tumefaciens* strain EHA101 in a like manner as described in Example 7.

10 Alternatively, the desaturase safflower clone may be prepared such that restriction sites flank the translation start and stop sites, as described in Example 8, with the following modification. PCR was carried out according to manufacturer's instructions except for the initial annealing  
15 of the oligonucleotides to the template. The reaction mix was heated to 90°C for 5 min, cooled to 37°C over a one hour period, kept at 37°C for 20 min and then subjected to standard PCR cycles. The PCR product was digested with *Pst*I and ligated to pUC8 (Vieira and Messing (1982) *Gene* 19:2359-  
20 268) digested with *Pst*I to produce pCGN3220. The *Nco*I/*Sac*I fragment of pCGN3220 containing the pUC8 vector and the 5' and 3' sequences of the safflower desaturase cDNA was gel purified and ligated to the gel-purified cloned *Nco*I/*Sac*I fragment from pCGN1894 (see Example 6). The resulting  
25 plasmid pCGN3222 contains safflower desaturase cDNA sequences partially from the cDNA clone and partially from the PCR. The regions obtained from the PCR were confirmed by DNA

sequencing as being identical to the original cloned sequence.

### *Expression Cassettes*

#### 5 Napin 1-2 pCGN1808 Expression Cassette

An expression cassette utilizing 5' upstream sequences and 3' downstream sequences obtainable from *B. campestris* napin gene can be constructed as follows.

A 2.7 kb *Xho*I fragment of napin 1-2 (Fig. 10 and SEQ ID NO: 29) containing 5' upstream sequences is subcloned into pCGN789 (a pUC based vector the same as pUC119 with the normal polylinker replaced by the synthetic linker - 5'GGAATTCGTCGACAGATCTCTGCAGCTCGAGGGATCCAAGCTT 3', SEQ ID NO: 41, (which represented the polylinker *Eco*RI, *Sal*I, *Bgl*II, *Pst*I, *Xho*I, *Bam*HI, *Hind*III) and results in pCGN940. The majority of the napin coding region of pCGN940 was deleted by digestion with *Sal*I and religation to form pCGN1800. Single-stranded DNA from pCGN1800 was used in an *in vitro* mutagenesis reaction (Adelman et al., *DNA* (1983) 2:183-193) using the synthetic oligonucleotide 5' GCTTGTTTCGCCATGGATATCTTCTGTATGTTC 3', SEQ ID NO: 42. This oligonucleotide inserted an *Eco*RV and an *Nco*I restriction site at the junction of the promoter region and the ATG start codon of the napin gene. An appropriate mutant was identified by hybridization to the oligonucleotide used for the mutagenesis and sequence analysis and named pCGN1801.

A 1.7 kb promoter fragment was subcloned from pCGN1801 by partial digestion with *EcoRV* and ligation to pCGN786 (a pCGN566 chloramphenicol based vector with the synthetic linker described above in place of the normal polylinker) cut with *EcoRI* and blunted by filling in with DNA Polymerase I Klenow fragment to create pCGN1802.

A 2.1 kb *SalI* fragment of napin 1-2 (Fig. 10 and SEQ ID NO: 29) containing 3' downstream sequences is subcloned into pCGN789 (described above) and results in pCGN941. pCGN941 is digested with *XhoI* and *HindIII* and the resulting approximately 1.6 kb of napin 3' sequences are inserted into *XhoI-HindIII* digested pCGN1802 to result in pCGN1803. In order to remove a 326 nucleotide *HindIII* fragment inserted opposite to its natural orientation, as a result of the fact that there are 2 *HindIII* sites in pCGN1803, the pCGN1803 is digested with *HindIII* and religated. Following religation, a clone is selected which now contains only 1.25 kb of the original 1.6 napin 3' sequence. This clone, pCGN1808 is the napin 1-2 expression cassette and contains 1.725 kb of napin promoter sequences and 1.265 kb of napin 3' sequence with the unique cloning sites *SalI*, *BglI*, *PstI* and *XhoI* in between.

#### Napin 1-2 pCGN3223 Expression Cassette

Alternatively, pCGN1808 may be modified to contain flanking restriction sites to allow movement of only the expression sequences and not the antibiotic resistance marker to binary vectors such as pCGN1557 (McBride and Summerfelt,



supra). Synthetic oligonucleotides containing *KpnI*, *NotI* and *HindIII* restriction sites are annealed and ligated at the unique *HindIII* site of pCGN1808, such that only one *HindIII* site is recovered. The resulting plasmid, pCGN3200 contains  
 5 unique *HindIII*, *NotI* and *KpnI* restriction sites at the 3'-end of the napin 3'-regulatory sequences as confirmed by sequence analysis.

The majority of the napin expression cassette is subcloned from pCGN3200 by digestion with *HindIII* and *SacI* and ligation to *HindIII* and *SacI* digested pIC19R (Marsh, et  
 10 al. (1984) *Gene* 32:481-485) to make pCGN3212. The extreme 5'-sequences of the napin promoter region are reconstructed by PCR using pCGN3200 as a template and two primers flanking the *SacI* site and the junction of the napin 5'-promoter and  
 15 the pUC backbone of pCGN3200 from the pCGN1808 construct.

The forward primer contains *ClaI*, *HindIII*, *NotI*, and *KpnI* ~~restriction~~ *restriction* sites as well as nucleotides 408-423 of the napin  
 B A 5'-sequence (from the *EcoRV* site) and the reverse primer contains the complement to napin sequences 718-739 which  
 20 include the unique *SacI* site in the 5'-promoter. The PCR was performed using a Perkin Elmer/Cetus thermocycler according to manufacturer's specifications. The PCR fragment is subcloned as a blunt-ended fragment into pUC8 (Vieira and Messing (1982) *Gene* 19:259-268) and digested with *HincII* to  
 25 give pCGN3217. Sequence of pCGN3217 across the napin insert verifies that no improper nucleotides were introduced by PCR. The napin 5-sequences in pCGN3217 are ligated to the

remainder of the napin expression cassette by digestion with *Cla*I and *Sac*I and ligation to pCGN3212 digested with *Cla*I and *Sac*I. The resulting expression cassette pCGN3221, is digested with *Hind*III and the napin expression sequences are gel purified away and ligated to pIC20H (Marsh, *supra*) digested with *Hind*III. The final expression cassette is pCGN3223, which contains in an ampicillin resistant background, essentially identical 1.725 napin 5' and 1.265 3' regulatory sequences as found in pCGN1808. The regulatory regions are flanked with *Hind*III, *Not*I and *Kpn*I restriction sites and unique *Sal*I, *Bgl*III, *Pst*I, and *Xho*I cloning sites are located between the 5' and 3' noncoding regions.

Desaturase sequences in sense or anti-sense orientation may be inserted into a napin expression cassette via the cloning sites. The resulting construct may be employed for plant transformation. For example, one of ordinary skill in the art could also use known techniques of gene cloning, mutations, insertion and repair to allow cloning of a napin expression cassette into any suitable binary vector, such as pCGN1557 (described in Example 7) or other similar vectors.

#### *Desaturase Expression*

The coding region of the safflower desaturase contained in pCGN3222 is cloned into the pCGN3223 napin cassette by digestion with *Xho*I and ligation to pCGN3223 digested with *Xho*I and *Sal*I. The resulting plasmid, pCGN3229 is digested with *Asp*718 and inserted in the binary vector pCGN1578

(McBride and Summerfelt (1990) *Plant Mol. Biol.* 14:269-276) at the unique Asp718 site. The resulting binary vector is pCGN3231 and contains the safflower desaturase coding sequences flanked by the napin 5' and 3' regulatory sequences as well as the plant selectable marker construct, 35s/NPTII/tml.

The resulting binary vector, pCGN3231, is transformed into *Agrobacterium* and utilized for plant transformation as described in Example 10. For Northern analysis, total RNA is isolated from day 21 and day 28 post-anthesis developing seed from plants transformed with pCGN3231. Five samples were analyzed at day 21 and two at day 28 post-anthesis. RNA was isolated by the method of Hughes and Galau (*Plant Mol. Biol. Reporter* (1988) 6: 253-257). Northern blot analysis was performed using a labeled 0.8 kb *Bgl*III fragment of pCGN1894 as a probe. Prehybridization and hybridization was at 42°C in 50% formamide, 10X Denhardt's solution, 5X SSC, 0.1% SDS, 5mM EDTA and 100ug/ml denatured salmon sperm DNA. Filters were washed at 55°C in 0.1 X SSC, 0.1% SDS. Under these conditions, the probe does not hybridize to the endogenous *Brassica* desaturase gene sequences. mRNA complementary to the safflower desaturase was detected in all the transgenic samples examined. More mRNA was present at day 28 than at day 21 post-anthesis and the highest level of RNA was seen in transgenic 3231-8. The total safflower desaturase mRNA level was estimated to be ~0.01% of the message at day 28 post-anthesis.

Western analysis (see below) gives a preliminary indication of increased protein in one transformant, 3231-8 in a *Brassica napus* cv. Delta plant. However, the Western analysis is complicated by two factors: 1. The presence of cross-reacting material at the same molecular weight as expected for the safflower desaturase. We believe this material is the endogenous *Brassica* desaturase. 2. The analysis of levels of protein expressed is also complicated by the normal developmental increase in the expression of desaturase protein during this time period. If the seeds examined are not at the precise developmental stage as the control seeds, quantitative differences in the amount of material seen may be simply due to the normal increase in the *Brassica* desaturase over this time period and not due to the expression of the safflower desaturase.

#### Western Analysis

Soluble protein is extracted from developing seeds of *Brassica* by homogenization with one volume (1ml/gram fresh weight) of buffer containing 20mM potassium phosphate, pH 6.8. The homogenate is clarified by centrifugation at 12,000 x g for 10 minutes. A second centrifugation is performed if necessary to provide a non-particulate supernatant.

Protein concentration of the extract is measured by the micromethod of Bradford (*Anal. Biochem.* (1976) 72:248-254). Proteins (20-60µg) are separated by denaturing electrophoresis by the method of Laemmli (*supra*), and are

transferred to nitrocellulose membrane by the method of Towbin et al. (*Proc. Nat. Acad Sci.* (1979) 76:4350-4354).

The nitrocellulose membrane is blocked by incubation at room temperature for 15 minutes or at 4°C overnight in Tris-  
5 buffered saline with Tween 20 (Polyoxyethylenesorbitan monolaurate) and "TTBS-milk", (TTBS = 20mM Tris-HCl, 500mM NaCl, 0.1% Tween 20 (v/v), pH 7.5; "TTBS-milk" = TTBS and 3% skim milk powder). The volume of liquid in all incubations with the nitrocellulose membrane is sufficient to cover the  
10 membrane completely. The membrane is then incubated for an additional 5 minutes in TTBS.

The nitrocellulose membrane is incubated for at least one hour with shaking at room temperature with rabbit anti-stearoyl-ACP desaturase antiserum that was diluted 5,000- or  
15 10,000-fold in "TTBS-milk". The rabbit anti-desaturase antiserum was commercially prepared from desaturase protein (purified as described in Example 1) by Berkeley Antibody Co. (Richmond, CA). The membrane is washed twice by shaking with TTBS for 5 minutes and then with deionized H<sub>2</sub>O for 30  
20 seconds.

The nitrocellulose membrane is incubated for at least 45 minutes at room temperature in a solution of "TTBS-milk" in which anti-rabbit IgG-alkaline phosphatase conjugate (Promega, Madison, WI) is diluted 7,500-fold. The membrane  
25 is washed twice in TTBS followed by deionized H<sub>2</sub>O, as described above.

The nitrocellulose membrane is equilibrated in buffer containing 100mM Tris-HCl, 100mM NaCl, 50mM MgCl<sub>2</sub>, pH 9.5, by shaking for 5 minutes. The color reaction is initiated by placing the nitrocellulose membrane into 50ml of the same buffer to which has been added 15mg p-nitroblue tetrazolium chloride and 7.5mg 5-bromo- 4 chloro- 3-indolyl phosphate toluidine salt (BioRad Labs; Richmond, CA). The color reaction is stopped by rinsing the nitrocellulose membrane with deionized H<sub>2</sub>O and drying it between filter papers.

#### 10 Fatty Acid Analysis

Analysis of developing seeds indicated no significant change in fatty acid (total seed lipid) composition of the transformed pCGN3231 Delta plants with respect to the control plants. This result is consistent with the low levels of safflower mRNA observed in transgenic plants as compared to levels of endogenous *Brassica* desaturase (Example 12).

However, subsequent fatty acid analysis of individual mature seeds of Delta plants containing the pCGN3231 construct showed an average of  $0.97 \pm 0.16\%$  stearate compared with an average of  $1.47 \pm 0.24\%$  obtained from seed testing of 2 different Delta control plants. Individual seeds show as little as .8% stearate and a saturate content (16:0 + 18:0) as low as 4.9%.

#### 25 Example 10

In this example, an *Agrobacterium*-mediated plant transformation is described. *Brassica napus* is exemplified.

The method is also useful for transformation of other *Brassica* species including *Brassica campestris*.

#### *Plant Material and Transformation*

5       Seeds of *Brassica napus* cv. Delta are soaked in 95% ethanol for 2 min, surface sterilized in a 1.0% solution of sodium hypochlorite containing a drop of Tween 20 for 45 min., and rinsed three times in sterile, distilled water. Seeds are then plated in Magenta boxes with 1/10th  
10       concentration of Murashige minimal organics medium (Gibco) supplemented with pyridoxine (50  $\mu\text{g/l}$ ), nicotinic acid (50  $\mu\text{g/l}$ ), glycine (200  $\mu\text{g/l}$ ), and 0.6% Phytagar (Gibco) pH 5.8. Seeds are germinated in a culture room at 22°C in a 16 h photoperiod with cool fluorescent and red light of intensity  
15       approximately 65  $\mu\text{Einsteins per square meter per second}$  ( $\mu\text{Em}^{-2}\text{s}^{-1}$ ).

      Hypocotyls are excised from 7 day old seedlings, cut into pieces approximately 4 mm in length, and plated on feeder plates (Horsch et al. 1985). Feeder plates are  
20       prepared one day before use by plating 1.0 ml of a tobacco suspension culture onto a petri plate (100x25 mm) containing about 30 ml MS salt base (Carolina Biological) 100 mg/l inositol, 1.3 mg/l thiamine-HCl, 200 mg  $\text{KH}_2\text{PO}_4$  with 3% sucrose, 2,4-D (1.0 mg/l), 0.6% Phytagar, and pH adjusted to  
25       5.8 prior to autoclaving (MS0/1/0 medium). A sterile filter paper disc (Whatman 3 mm) is placed on top of the feeder layer prior to use. Tobacco suspension cultures are

subcultured weekly by transfer of 10 ml of culture into 100 ml fresh MS medium as described for the feeder plates with 2,4-D (0.2 mg/l), Kinetin (0.1 mg/l). All hypocotyl explants are preincubated on feeder plates for 24 h. at 22°C in  
 5 continuous light of intensity  $30 \mu\text{Em}^{-2}\text{s}^{-1}$  to  $65 \mu\text{Em}^{-2}\text{s}^{-1}$ .

Single colonies of *A. tumefaciens* strain EHA101 containing a binary plasmid are transferred to 5 ml MG/L broth and grown overnight at 30°C. Per liter, MG/L broth contains 5g mannitol, 1 g L-glutamic acid or 1.15 g sodium  
 10 glutamate, 0.25 g  $\text{KH}_2\text{PO}_4$ , 0.10 g NaCl, 0.10 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1 mg biotin, 5 g tryptone, and 2.5 g yeast extract, and the broth is adjusted to pH 7.0. Hypocotyl explants are immersed in 7-12 ml MG/L broth with bacteria diluted to  $1 \times 10^8$  bacteria/ml and after 10-20 min. are placed onto feeder  
 15 plates. After 48 h of co-incubation with *Agrobacterium*, the hypocotyl explants are transferred to B5 0/1/0 callus induction medium which contains filter sterilized carbenicillin (500 mg/l, added after autoclaving) and kanamycin sulfate (Boehringer Mannheim) at concentrations of  
 20 25 mg/l.

After 3-7 days in culture at  $65 \mu\text{Em}^{-2}\text{s}^{-1}$  to  $75 \mu\text{Em}^{-2}\text{s}^{-1}$  continuous light, callus tissue is visible on the cut surface and the hypocotyl explants are transferred to shoot induction medium, B5BZ (B5 salts and vitamins supplemented with 3 mg/l  
 25 benzylaminopurine, 1 mg/l zeatin, 1% sucrose, 0.6% Phytagar and pH adjusted to 5.8). This medium also contains carbenicillin (500 mg/l) and kanamycin sulfate (25 mg/l).



Hypocotyl explants are subcultured onto fresh shoot induction medium every two weeks.

Shoots regenerate from the hypocotyl calli after one to three months. Green shoots at least 1 cm tall are excised from the calli and placed on medium containing B5 salts and vitamins, 1% sucrose, carbenicillin (300 mg/l), kanamycin sulfate (50 mg/l) and 0.6% Phytagar) and placed in a culture room with conditions as described for seed germination.

After 2-4 weeks shoots which remain green are cut at the base and transferred to Magenta boxes containing root induction medium (B5 salts and vitamins, 1% sucrose, 2 mg/l indolebutyric acid, 50 mg/l kanamycin sulfate and 0.6% Phytagar). Green rooted shoots are tested for NPT II activity.

#### Example 11

In this example, a DNA-bombardment plant transformation is described. Peanut transformation is exemplified.

DNA sequences of interest may be introduced as expression cassettes, comprising at least a promoter region, a gene of interest, and a termination region, into a plant genome via particle bombardment as described in European Patent Application 332 855 and in co-pending application USSN 07/225,332, filed July 27, 1988.

Briefly, tungsten or gold particles of a size ranging from 0.5 $\mu$ M-3 $\mu$ M are coated with DNA of an expression cassette.

This DNA may be in the form of an aqueous mixture or a dry DNA/particle precipitate.

Tissue used as the target for bombardment may be from cotyledonary explants, shoot meristems, immature leaflets, or  
5 anthers.

The bombardment of the tissue with the DNA-coated particles is carried out using a Biolistics™ particle gun (Dupont; Wilmington, DE). The particles are placed in the barrel at variable distances ranging from 1cm-14cm from the  
10 barrel mouth. The tissue to be bombarded is placed beneath the stopping plate; testing is performed on the tissue at distances up to 20 cm. At the moment of discharge, the tissue is protected by a nylon net or a combination of nylon nets with mesh ranging from 10μM to 300μM.

15 Following bombardment, plants may be regenerated following the method of Atreya, et al., (*Plant Science Letters* (1984) 34:379-383). Briefly, embryo axis tissue or cotyledon segments are placed on MS medium (Murashige and Skoog, *Physio. Plant.* (1962) 15:473) (MS plus 2.0 mg.l 6-  
20 benzyladenine (BA) for the cotyledon segments) and incubated in the dark for 1 week at  $25 \pm 2^{\circ}\text{C}$  and are subsequently transferred to continuous cool white fluorescent light (6.8 W/m<sup>2</sup>). On the 10th day of culture, the plantlets are transferred to pots containing sterile soil, are kept in the  
25 shade for 3-5 days and finally moved to greenhouse.

The putative transgenic shoots are rooted. Integration of exogenous DNA into the plant genome may be confirmed by various methods known to those skilled in the art.

## 5 **Example 12**

This example describes methods to obtain desaturase cDNA clones from other plant species using the DNA from the *C. tinctorius*  $\Delta$ -9 desaturase clone as the probe.

### 10 *Isolation of RNA for Northern Analysis*

Poly(A)<sup>+</sup> RNA is isolated from *C. tinctorius* embryos collected at 14-17 days post-anthesis and *Simmondsia chinensis* embryos as described in Example 5.

Total RNA is isolated from days 17-18 days post-anthesis

15 *Brassica campestris* embryos by an RNA miniprep preparation technique (Scherer and Knauf, *Plant Mol. Biol.* (1987) 9:127-134). Total RNA is isolated from *R. communis* immature endosperm of about 14-21 days post-anthesis by a method described by Halling, et al. (*Nucl. Acids Res.* (1985)

20 13:8019-8033). Total RNA is isolated from 10 g each of young leaves from *B. campestris*, *B. napus*, and *C. tinctorius*, by extraction of each sample in 5 ml/g tissue of 4 M guanidine thiocyanate buffer as described by Colbert et al. (*Proc. Nat. Acad. Sci.* (1983) 80:2248-2252). Total RNA is also isolated

25 from immature embryos of *Cuphea hookeriana* by extraction as above in 10 ml/g tissue.

Total RNA is isolated from immature embryos of California bay (*Umbellularia californica*) by an adaptation of the method of Lagrimini et al. (*Proc. Nat. Acad. Sci.* (1987) 84:7542-7546). Following homogenization in grinding buffer (2.5 ml/g tissue) as described, RNA is precipitated from the aqueous phase by addition of 1/10 volume 3 M sodium acetate and 2 volumes ethanol, followed by freezing at -80°C for 30 minutes and centrifugation at 13,000 x g for 20 minutes. The pellets are washed with 80% ethanol and centrifugation is repeated as above. The pellets are resuspended in water, two volumes of 4 M LiCl are added, and the samples are placed at -20°C overnight. Samples are centrifuged as above and the pellets washed with 80% ethanol. Ethanol precipitation is repeated as above.

Total RNA is further purified from *B. campestris*, *B. napus*, and *C. tinctorius* leaves, and from *C. tinctorius*, *B. campestris*, California bay, and jojoba, and from *R. communis* immature endosperm, by removing polysaccharides on a 0.25 g Sigma Cell 50 cellulose column. The RNA is loaded onto the column in 1 ml of loading buffer (20 mM Tris-HCl pH 7.5, 0.5M NaCl, 1mM EDTA, 0.1% SDS), eluted with loading buffer, and collected in 500 µl fractions. Ethanol is added to the samples to precipitate the RNA. The samples are centrifuged, and the pellets resuspended in sterile distilled water, pooled, and again precipitated in ethanol. The sample is centrifuged, and the resulting RNA is subjected to oligo(dT)-cellulose chromatography to enrich for poly(A)+ RNA as

described by Maniatis et al. (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York (1982)).

Poly(A)+ RNA is also purified from total *Cuphea hookeriana* RNA by oligo(dT)-cellulose chromatography.

5        *Northern Analysis Using C. tinctorius Desaturase Clone:*  
2.5 µg of poly(A)+ RNA from each of the above described  
poly(A)+ samples from immature embryos of jojoba, *Cuphea*  
*hookeriana*, California bay, *Brassica campestris*, and *C.*  
*tinctorius*, from immature endosperm of *R. communis*, and from  
10 leaves of *C. tinctorius*, *B. campestris*, and *B. napus* are  
electrophoresed on formaldehyde/agarose gels (Fourney et al.,  
*Focus* (1988) 10:5-7) and transferred to a Hybond-C extra  
(Amersham, Arlington Heights, IL) filter according to  
manufacturer's specifications. The filter is prehybridized  
15 for four hours and hybridized overnight at 42°C in a roller  
bottle containing 10 ml of hybridization buffer (1 M NaCl, 1%  
SDS, 50% formamide, 0.1 mg/ml denatured salmon sperm DNA) in  
a Hybridization Incubator, model 1040-00-1 (Robbins  
Scientific Corporation, Sunnyvale, CA). The probe used in  
20 the hybridization is a gel-isolated *Bgl*III fragment of the Δ-9  
desaturase clone that is labeled with <sup>32</sup>P-dCTP using a BRL  
(Gaithersburg, MD) nick-translation kit, following  
manufacturer's instructions. The blot is washed three times  
for 20 minutes each in 2X SSC, 0.5% SDS at 55°C. The blot is  
25 exposed at -80°C, with a Dupont Cronex intensifying screen,  
to X-ray film for four days.

The autoradiograph shows that the *C. tinctorius* desaturase gene is expressed in both immature embryos and leaves of *C. tinctorius*, although the level of expression is considerably higher in embryos than in leaves. The  
5 autoradiograph also shows hybridization of the *C. tinctorius* desaturase clone to mRNA bands of a similar size in immature embryos from jojoba and California bay, and immature endosperm from *R. communis*. Hybridization is also detectable in RNA from *B. campestris* embryos upon longer exposure of the  
10 filter to X-ray film.

*R. communis* cDNA Library Construction: A plant seed cDNA library may be constructed from poly(A)+ RNA isolated from *R. communis* immature endosperm as described above. The plasmid cloning vector pCGN1703, and cloning method are as  
15 described in Example 5. The *R. communis* endosperm cDNA bank contains approximately  $2 \times 10^6$  clones with an average cDNA insert size of approximately 1000 base pairs.

The *R. communis* immature endosperm cDNA bank is moved into the cloning vector lambda gt22 (Stratagene Cloning  
20 Systems) by digestion of total cDNA with NotI and ligation to lambda gt22 DNA digested with NotI. The resulting phage are packaged using a commercially available kit and titered using *E. coli* strain LE392 (Stratagene Cloning Systems, La Jolla, CA). The titer of the resulting library was approximately  
25  $1.5 \times 10^7$  pfu/ml.

*R. communis* cDNA Library Screen: The library is plated on *E. coli* strain LE392 at a density of approximately 25,000

pfu/150mm NZY plate to provide approximately 50,000 plaques for screening. Phage are lifted in duplicate on to NEN (Boston, MA.) Colony/Plaque Screen filters as described in Example 5. Following prehybridization at 42°C in 25 ml of hybridization buffer (1 M NaCl, 1% SDS, 50% formamide, 0.1 mg/ml denatured salmon sperm DNA) filters are hybridized overnight with a gel-purified 520 base pair *Bgl*III fragment of the *C. tinctorius* desaturase clone (Figure 7A) that is radiolabeled with <sup>32</sup>P-dCTP using a BRL (Gaithersburg, MD) Nick Translation System. Filters are washed three times for 20 minutes each in 2X SSC, 0.5% SDS at 55°C in a shaking water bath. Filters are exposed to X-ray film overnight at -80°C with a Dupont Cronex intensifying screen.

Clones are detected by hybridization on duplicate filters with the *C. tinctorius* desaturase cDNA fragment and plaque purified. During plaque purification, it was observed that larger plaques were obtained when *E. coli* strain Y1090 (Young, R.A. and Davis, R.W., *Proc. Natl. Acad. Sci. USA* (1983) 80:1194) was used as the host strain. This strain was thus used in subsequent plaque purification steps. Phage DNA is prepared from the purified clones as described by Grossberger (*NAR* (1987) 15:6737) with the following modification. The proteinase K treatment is replaced by the addition of 10% SDS and a 10 minute incubation at room temperature. Recovered phage DNA is digested with *Eco*RI, religated at low concentration, and transformed into *E. coli* DH5α (BRL; Gaithersburg, MD) cells to recover plasmids

containing cDNA inserts in pCGN1703. Minipreparation DNA (Maniatis et al., supra) is prepared from the clones and DNA sequence is determined as described above. Partial nucleotide sequence of the cDNA insert of a *R. communis* desaturase clone pCGN3230 is presented in Figure 3A and SEQ ID NO: 14. The complete nucleotide sequence of this clone is presented in Fig. 3B and as SEQ ID NO: 15.

*Northern Analysis Using R. communis Desaturase Clone:*

Total RNA for Northern analysis is isolated from tobacco leaves by the method of Ursin et al. (*Plant Cell* (1989) 1:727-736), petunia and tomato leaves by the method of Ecker and Davis (*Proc.Nat.Acad.Sci.* (1987) 84:5202-5206), and corn leaves by the method of Turpen and Griffith (*Biotechniques* (1986) 4:11-15). Total RNA samples from tobacco, corn, and tomato leaves are enriched for poly(A)+ RNA by oligo(dT)-cellulose chromatography as described by Maniatis et al. (supra).

Poly(A)+ RNA samples from tomato leaves (4 µg) and corn and tobacco leaves (1 µg each), and total RNA from petunia leaves (25 µg) are electrophoresed on a formaldehyde/agarose gel as described by Shewmaker et al. (*Virology* (1985) 140:281-288). Also electrophoresed on this gel are poly(A)+ RNA samples isolated from *B. campestris* day 17-19 embryos and *B. campestris* leaves (2 µg each), immature embryos from *C. tinctorius*, bay, and jojoba (1 µg each), and *R. communis* endosperm (1 µg). The isolation of these poly(A)+ RNA samples is described above for the Northern analysis using *C.*



*tinctorius* desaturase cDNA as probe. The RNA is transferred to a nitrocellulose filter as described by Shewmaker et al. (supra) and prehybridized and hybridized at 42°C in 50% formamide, 10X Denhardt's solution (described in Maniatis et al. (supra)), 5X SSC, 0.1% SDS, 5 mM EDTA, 100 ug/ml denatured salmon sperm DNA, and 10% dextran sulfate (in hybridization buffer only). The probe for hybridization is the <sup>32</sup>P-labeled (BRL Nick Translation Kit) 1.7 kb SalI insert of pCGN3230 that has been gel-purified from miniprep preparation DNA. The filter is washed following hybridization for 30 minutes in 2X SSC, 0.1% SDS at 42°C and at 50°C twice for 15 minutes each. The filter is exposed to X-ray film overnight at -80°C with a Dupont Cronex intensifying screen.

The autoradiograph shows hybridization of the *R. communis* desaturase clone to mRNA bands of a similar size in immature embryos from *B. campestris*, California bay, and *C. tinctorius*, and also in corn leaves and *R. communis* endosperm.

*B. campestris* Embryo cDNA Library Construction: Total RNA is isolated from 5 g of *B. campestris* cv. R500 embryos obtained from seeds harvested at days 17-19 post-anthesis. RNA is extracted in 25 mls of 4 M guanidine thiocyanate buffer as described by Colbert et al. (PNAS (1983) 80:2248-2252). Polysaccharides are removed from the RNA sample by resuspending the pellet in 6 ml of 1X TE (10 mM Tris/1 mM EDTA pH 8), adding potassium acetate to a concentration of 0.05M, and adding one half volume of ethanol. The sample is placed on ice for 60 minutes and centrifuged for 10 minutes at 3000 x g. RNA is

precipitated from the supernatant by adding sodium acetate to a concentration of 0.3 M followed by the addition of two volumes of ethanol. RNA is recovered from the sample by centrifugation at 12,000 x g for 10 minutes and yield calculated by UV

5 spectrophotometry. Two mg of the total RNA is further purified by removing polysaccharides on a 0.25 g Sigma Cell 50 cellulose column, as described above, and is also enriched for poly(A)+ RNA by oligo(dT)-cellulose chromatography as described above.

A *B. campestris* day 17-19 post anthesis embryo cDNA  
10 library is constructed in plasmid vector pCGN1703 as described in Example 5, using 5 ug of the above described poly(A)+ RNA. The library, which consists of approximately  $1.5 \times 10^5$  transformants, is amplified by plating and scraping colonies, and is stored as frozen *E. coli* cells in 10% DMSO  
15 at  $-80^{\circ}\text{C}$ . DNA is isolated from a portion of the amplified library by scaling up the alkaline lysis technique of Birnboim and Doly (*Nucleic Acids Res.* (1979) 7:1513), and purified by CsCl centrifugation. Library DNA is digested with *EcoRI* and is cloned into *EcoRI*-digested bacteriophage  
20 lambda gt10 (Stratagene; La Jolla, CA) DNA. The DNA is packaged using Gigapack II Gold *in vitro* packaging extracts (Stratagene; La Jolla, CA) according to manufacturer's specifications. The titer of the phage stock, determined by dilution plating of phage in *E. coli* C600 hfl- cells (Huynh,  
25 *et al.*, *DNA Cloning. Volume 1.* Eds. Gover, D.M. (1985) IRL Press Limited: Oxford, England, pp. 56,110), is  $6 \times 10^6$  pfu per ml.

*B. campestris* cDNA Library Screen: The library is plated on *E. coli* strain C600 hfl- at a density of approximately 30,000 pfu/150mm NZY plate to provide approximately 120,000 plaques for screening. Phage are  
5 lifted in duplicate on to NEN (Boston, MA.) Colony/Plaque Screen filters as described in Example 5. Filters are prehybridized and hybridized with the <sup>32</sup>P-labeled fragment of pCGN3230 as described above for the Northern hybridization. Filters are washed for 30 minutes in 2X SSC, 0.1% SDS at 50°C  
10 and at 55°C twice for 15 minutes each. Filters are exposed to X-ray film overnight at -80°C with a Dupont Cronex intensifying screen.

Clones are detected by hybridization on duplicate filters to the *R. communis* desaturase cDNA fragment and  
15 plaque purified. During plaque purification, the probe used was a gel-purified 1.4 kb SstI fragment of pCGN3230 which lacks the poly(A)+ tail. As described above, phage DNA is isolated from purified lambda clones, digested with EcoRI, ligated, and transformed to *E. coli* DH5α cells.  
20 Miniprep preparation DNA is prepared and partial DNA sequence determined as described above. Partial DNA sequences of two clones, pCGN3235 and pCGN3236, are presented in Figure 4A (SEQ ID NO: 17) and 4B (SEQ ID NO: 18), respectively. Initial DNA sequence analysis of the 3' regions of these  
25 clones indicates that pCGN3236 and pCGN3235 are cDNA clones from the same gene. pCGN3236 is a shorter clone than pCGN3235, which appears to contain the entire coding region

of the *B. campestris* desaturase gene. The complete nucleotide sequence of pCGN3235 is presented in Figure 4C and SEQ ID NO: 19.

*Desaturase Gene Analysis: Southern and Northern*

5 analyses of Brassica species are conducted to determine the number of genes which encode the Brassica desaturase clone, pCGN3235 in *B. campestris*, *B. oleracea*, and *B. napus*, and the timing of expression of the gene in *B. campestris* developing seeds. DNA is isolated from leaves of each of the above-  
10 named Brassica species by the method of Bernatzky and Tanksley (*Theor. Appl. Genet.* (1986) 72:314-321). DNA from each of the species is digested with restriction endonucleases EcoRI and XbaI (10 ug/digest), electrophoresed in a 0.7% agarose gel, and transferred to a nitrocellulose  
15 filter (Maniatis et al., *supra*). The filter is prehybridized and hybridized at 42°C (as described above for Northern analysis using *R. communis* desaturase clone) with a <sup>32</sup>P-labeled (nick translation) gel-isolated HindIII/PvuII fragment of pCGN3235 (Fig. 7C). The filter is washed  
20 following overnight hybridization, for 30 minutes at 55°C in 1X SSC, 0.1% SDS, followed by two 15 minute washed at 55°C in 0.1X SSC, 0.1% SDS.

The autoradiograph indicates that the Brassica desaturase is encoded by a small gene family consisting of  
25 about two genes in *B. campestris* and *B. oleracea*, and about four genes in *B. napus*.

The timing of expression of the desaturase gene during seed development is determined by Northern analysis. RNA is isolated from immature seeds of *B. campestris* cv. R500 collected at 11, 13, 15, 17, 19, 21, 25, 30, 35, and 40 days post-anthesis. Total RNA is isolated as described by Scherer and Knauf (*Plant Mol. Biol.* (1987) 9:127-134). Twenty five micrograms of RNA from each time point are electrophoresed through a formaldehyde-containing 1.5% agarose gel as described by Shewmaker, et al. (*supra*) and blotted to nitrocellulose (Thomas, *Proc. Nat. Acad. Sci.* (1980) 77:5201-5205). The blot is pre-hybridized and hybridized at 42°C with the <sup>32</sup>P-labeled HindIII/PvuII fragment of pCGN3235 as described above. The filter is washed following overnight hybridization, for 30 minutes at 55°C in 1X SSC, 0.1% SDS, followed by two 15 minute washed at 55°C in 0.1X SSC, 0.1% SDS.

The autoradiograph indicates that the desaturase gene is expressed in *B. campestris* developing seeds beginning at about day 19 and through about day 30, with maximal expression at day 25. By a similar Northern analysis, the level of desaturase mRNA in developing *Brassica napus* seeds (day 21) was estimated to be approximately 1% of the total mRNA.

*Isolation of Other Desaturase Gene Sequences:* cDNA libraries may be constructed as described above and genomic libraries can be constructed from DNA from various sources using commercially available vectors and published DNA

isolation, fractionation, and cloning procedures. For example, a *B. campestris* genomic library can be constructed using DNA isolated according to Scofield and Crouch (J.Biol.Chem. (1987) 262:12202-12208) that is digested with  
5 BamHI and fractionated on sucrose gradients (Maniatis et al., supra), and cloned into the lambda phage vector LambdaGem-11 (Promega; Madison, WI) using cloning procedures of Maniatis et al. (supra).

cDNA and genomic libraries can be screened for  
10 desaturase cDNA and genomic clones, respectively, using published hybridization techniques. Screening techniques are described above for screening libraries with DNA fragments. Libraries may also be screened with synthetic oligonucleotides, for example using methods described by  
15 Berent et al. (BioTechniques (1985) 3:208-220). Probes for the library screening can be prepared by PCR, or from the sequences of the desaturase clones provided herein. Oligonucleotides prepared from the desaturase sequences may be used, as well as longer DNA fragments, up to the entire  
20 desaturase clone.

For example, jojoba polyadenylated RNA is used to construct a cDNA library in the cloning vector  $\lambda$ ZAPII/EcoRI (Stratagene, San Diego, CA). RNA is isolated from jojoba embryos collected at 80-90 days post-anthesis by isolating  
25 polyribosomes using a method initially described by Jackson and Larkins (Plant Physiol. (1976) 57:5-10) and modified by Goldberg et al. (Developmental Biol. (1981) 83:201-217).

Polysaccharide contaminants in the polyribosomal RNA preparation are removed by running the RNA over a cellulose column (Sigma-cell 50) in high salt buffer (0.5M NaCl, 20mM Tris pH 7.5, 1mM EDTA, 0.1% SDS). The contaminant binds to the column and the RNA is collected in the eluant. The eluant fractions are pooled and the RNA is ethanol precipitated. The precipitated total RNA is then resuspended in a smaller volume and applied to an oligo d(T) cellulose column to isolate the polyadenylated RNA.

10 The library is constructed using protocols, DNA and bacterial strains as supplied by the manufacturer. Clones are packaged using Gigapack Gold packaging extracts (Stratagene), also according to manufacturer's recommendations. The cDNA library constructed in this manner contains approximately  $1 \times 10^6$  clones with an average cDNA insert size of approximately 400 base pairs.

The jojoba library is plated on *E. coli* XL1-Blue (Stratagene) at a density of approximately 5000pfu/150mm plate to provide approximately 60,000 plaques for screening.

20 Phage are lifted onto duplicate nylon membrane filters as described previously. Filters are prehybridized at 42°C in a hybridization buffer containing 40% formamide, 10X Denhardt's solution, 5X SSC, 0.1% SDS, 50mM EDTA, and 100µg/ml denatured salmon sperm DNA. Hybridization is at 42°C in the same

25 buffer with added nick translated (BRL Nick Translation System) 520 bp *Bgl*III fragment of the *C. tinctorius* desaturase

clone described previously. Filters are washed at 50°C in 2X SSC and exposed to X-ray film overnight.

Desaturase clones are detected by hybridization on duplicate filters with the *C. tinctorius* cDNA fragment and plaque-purified. Positive clones are recovered as plasmids in *E. coli* following manufacturer's directions and materials for *in vivo* excision. Partial, preliminary DNA sequence of a clone, 3-1, is determined and the corresponding amino acid sequence is translated in three frames. In this manner, homology to the *C. tinctorius* desaturase cDNA clone is detected in one reading frame. The preliminary DNA sequence of this jojoba desaturase cDNA fragment is shown in Figure 5 (SEQ ID NO: 43). Also shown is the corresponding translated amino acid sequence in the reading frame having *C. tinctorius* desaturase homology. The jojoba cDNA fragment is approximately 75% homologous at the DNA level and approximately 79% homologous at the amino acid level compared to sequence of the *C. tinctorius* desaturase in this region.

### 20 **Example 13**

Antisense constructs are described which allow for transcription of a reverse copy of the *B. campestris* desaturase cDNA clone in the 5' to 3' orientation of transcription. In a similar fashion, anti-sense constructs can be prepared to reduce desaturase levels in other plant hosts.



*Preferential Expression of Antisense Constructs in Embryos*

In order to reduce the transcription of a desaturase gene in embryos of *B. napus* or *B. campestris*, constructs may be prepared which allow for production of antisense copies of the desaturase cDNA preferentially in the embryos. Promoter sequences which are desirable to obtain this pattern of expression include, but are not limited to, the ACP, Bce4, and napin 1-2 expression cassettes described in Examples 7, 8, and 9, respectively. It also may be desirable to control the expression of reverse copies of the desaturase cDNA under two different promoters in the same transformed plant to provide for a broader timing of expression of the antisense desaturase DNA. For example, expression from the ACP promoter may begin and end earlier than expression from the napin promoter. Thus, expressing the reverse desaturase from both promoters may result in the production of the antisense strand of DNA over a longer period of embryo development.

An example of expression of an antisense desaturase gene preferentially in the embryos is provided below. Similar constructs containing the same or a different fragment of the desaturase gene and any of the promoters described above, as well as other promoter regions which may be useful, may also be prepared using gene cloning, insertion, mutation and repair techniques well known to those of ordinary skill in the art.

A. Antisense Desaturase Expression from the ACP Promoter

Construction of pCGN3239 is as follows:

pCGN3235 (Example 12) is digested with *PvuII* and *HindIII* and the *HindIII* sticky ends are filled in with Klenow in the presence of 200  $\mu$ M dNTPs. The 1.2 kb *PvuII/HindIII* fragment containing the desaturase coding sequence is gel purified and ligated in the antisense orientation into *EcoRV*-digested pCGN1977 (ACP expression cassette; described in Example 7) to create pCGN3238. The 4.2 kb *XbaI/Asp718* fragment of pCGN3238 containing the antisense desaturase in the ACP cassette is transferred into *XbaI/Asp718*-digested pCGN1557 (binary transformation vector; described in Example 7) to create pCGN3239.

B. Antisense Desaturase Expression From The Napin Promoter

Construction of pCGN3240 is as follows: pCGN3235 is digested with *PvuII* and *HindIII*, the sticky ends are blunted, and the resulting fragment is inserted in an anti-sense orientation into pCGN3223 which has been digested with *SalI* and blunted with Klenow enzyme. The resulting plasmid, pCGN3240 will express an anti-sense desaturase RNA from the napin promoter cassette.

C. Antisense Desaturase Expression From a Dual Promoter Cassette

Construction of pCGN3242 is as follows: An *Asp718* fragment of pCGN3240 containing the napin 5' and 3' regions surrounding the desaturase sequences is inserted into the

Asp718 site of pCGN3239 (a binary vector containing an ACP promoter, antisense desaturase construct) to create pCGN3242.

*Constitutive Transcription*

5 A. Binary Vector Construction

1. Construction of pCGP291.

The *KpnI*, *BamHI*, and *XbaI* sites of binary vector pCGN1559 (McBride and Summerfelt, *Pl.Mol.Biol.* (1990) 14: 269-276) are removed by Asp718/*XbaI* digestion followed by blunting the ends and recircularization to produce pCGP67. The 1.84 kb *PstI/HindIII* fragment of pCGN986 containing the 35S promoter-tml3' cassette is inserted into *PstI/HindIII* digested pCGP67 to produce pCGP291.

2. Construction of pCGN986.

15 The 35S promoter-tml3' expression cassette, pCGN986, contains a cauliflower mosaic virus 35S (CaMV35) promoter and a T-DNA tml 3'-region with multiple restriction sites between them. pCGN986 is derived from another cassette, pCGN206, containing a CaMV35S promoter and a different 3' region, the CaMV region VI 3'-end. The CaMV 35S promoter is cloned as an AluI fragment (bp 7144-7734) (Gardner et. al., *Nucl.Acids Res.* (1981) 9:2871-2888) into the *HincII* site of M13mp7 (Messing, et. al., *Nucl.Acids Res.* (1981) 9:309-321) to create C614. An *EcoRI* digest of C614 produced the *EcoRI* fragment from C614 containing the 35S promoter which is 25 cloned into the *EcoRI* site of pUC8 (Vieira and Messing, *Gene* (1982) 19:259) to produce pCGN147.

pCGN148a containing a promoter region, selectable marker (KAN with 2 ATG's) and 3' region, is prepared by digesting pCGN528 with *Bgl*II and inserting the *Bam*HI-*Bgl*II promoter fragment from pCGN147. This fragment is cloned into the  
5 *Bgl*II site of pCGN528 so that the *Bgl*II site is proximal to the kanamycin gene of pCGN528.

The shuttle vector used for this construct, pCGN528, is made as follows: pCGN525 is made by digesting a plasmid containing Tn5 which harbors a kanamycin gene (Jorgenson et.  
10 al., *Mol. Gen. Genet.* (1979) 177:65) with *Hind*III-*Bam*HI and inserting the *Hind*III-*Bam*HI fragment containing the kanamycin gene into the *Hind*III-*Bam*HI sites in the tetracycline gene of pACYC184 (Chang and Cohen, *J. Bacteriol.* (1978) 134:1141-1156). pCGN526 was made by inserting the *Bam*HI fragment 19  
15 of pTiA6 (Thomashow et. al., *Cell* (1980) 19:729-739), modified with *Xho*I linkers inserted into the *Sma*I site, into the *Bam*HI site of pCGN525. pCGN528 is obtained by deleting the small *Xho*I fragment from pCGN526 by digesting with *Xho*I and religating.

20 pCGN149a is made by cloning the *Bam*HI-kanamycin gene fragment from pMB9KanXXI into the *Bam*HI site of pCGN148a. pMB9KanXXI is a pUC4K variant (Vieira and Messing, *Gene* (1982) 19:259-268) which has the *Xho*I site missing, but contains a functional kanamycin gene from Tn903 to allow for  
25 efficient selection in *Agrobacterium*.

pCGN149a is digested with *Hind*III and *Bam*HI and ligated to pUC8 digested with *Hind*III and *Bam*HI to produce pCGN169.

This removes the Tn903 kanamycin marker. pCGN565 and pCGN169 are both digested with *Hind*III and *Pst*I and ligated to form pCGN203, a plasmid containing the CaMV 35S promoter and part of the 5'-end of the Tn5 kanamycin gene (up to the *Pst*I site, Jorgenson et al., (1979), supra). A 3'-regulatory region is added to pCGN203 from pCGN204, an *Eco*RI fragment of CaMV (bp 408-6105) containing the region VI 3' cloned into pUC18 (Yanisch-Perron, et al., *Gene* (1985) 33:103-119) by digestion with *Hind*III and *Pst*I and ligation. The resulting cassette, pCGN206, is the basis for the construction of pCGN986.

The pTiA6 T-DNA tml 3'-sequences are subcloned from the *Bam*19 T-DNA fragment (Thomashow et al., (1980) supra) as a *Bam*HI-*Eco*RI fragment (nucleotides 9062 to 12,823, numbering as in Barker et al., *Plant Mol. Biol.* (1982) 2:335-350) and combined with the pACYC184 (Chang and Cohen (1978), supra) origin of replication as an *Eco*RI-*Hind*III fragment and a gentamycin resistance marker (from plasmid pLB41), obtained from D. Figurski) as a *Bam*HI-*Hind*III fragment to produce pCGN417.

The unique *Sma*I site of pCGN417 (nucleotide 11,207 of the *Bam*19 fragment) is changed to a *Sac*I site using linkers and the *Bam*HI-*Sac*I fragment is subcloned into pCGN565 to give pCGN971. The *Bam*HI site of pCGN971 is changed to an *Eco*RI site using linkers. The resulting *Eco*RI-*Sac*I fragment containing the tml 3' regulatory sequences is joined to pCGN206 by digestion with *Eco*RI and *Sac*I to give pCGN975. The small part of the Tn5 kanamycin resistance gene is

deleted from the 3'-end of the CaMV 35S promoter by digestion with *Sal*I and *Bgl*II, blunting the ends and ligation with *Sal*I linkers. The final expression cassette pCGN986 contains the CaMV 35S promoter followed by two *Sal*I sites, an *Xba*I site, *Bam*HI, *Sma*I, *Kpn*I and the tml 3' region (nucleotides 11207-9023 of the T-DNA).

#### B. Insertion of Desaturase Sequence

The 1.6 kb *Xba*I fragment from pCGN3235 containing the desaturase cDNA is inserted in the antisense orientation into the *Xba*I site of pCGP291 to produce pCGN3234.

#### *Plant Transformation*

The binary vectors containing the expression cassette and the desaturase gene are transformed into *Agrobacterium tumefaciens* strain EHA101 (Hood, et al., *J. Bacteriol.* (1986) 168:1291-1301) as per the method of Holsters, et al., *Mol. Gen. Genet.* (1978) 163:181-187. Transformed *B. napus* and/or *Brassica campestris* plants are obtained as described in Example 10.

#### *Analysis of Transgenic Plants*

##### A. Analysis of pCGN3242 Transformed *Brassica campestris* cv. Tobin Plants

Due to the self-incompatibility of *Brassica campestris* cv. Tobin, individual transgenic plants are pollinated using non-transformed Tobin pollen. Because of this, the T2 seeds of a transgenic plant containing the antisense desaturase at

one locus would be expected to segregate in a 1:1 ratio of transformed to non-transformed seed. The fatty acid composition (total seed lipids) of ten individual seeds collected at 26 days post-anthesis from several pCGN3242-  
 5 transformed plants and one non-transformed control was analyzed by gas chromatography according to the method of Browse, et al., *Anal. Biochem.* (1986) 152:141-145. One transformant, 3242-T-1, exhibits a fatty acid composition that differed distinctly from controls on preliminary  
 10 analysis. The control Tobin seeds contained an average of 1.8% 18:0 (range 1.5% - 2.0%) and 52.9% 18:1 (range 48.2% - 57.1%). T2 seeds of 3242-T-1 segregated into two distinct classes. Five seeds contained levels of 18:0 ranging from 1.3% to 1.9% and levels of 18:1 ranging from 42.2% to 58.3%.  
 15 The other five seeds contained from 22.9% to 26.3% 18:0 and from 19.9% to 26.1% 18:1.

Analysis of individual mature seeds containing pCGN3242 in T2 seed yielded seed having up to 45% stearate by weight. No changes in the level of palmitate, the precursor to  
 20 stearate, <sup>are</sup> ~~are~~ observed. Increased percentages of 18:3 ~~and~~ low, but increased levels of long chain (>18 carbon) <sup>A</sup> saturated fatty acids are seen. Reductions in the average total oil content observed in these seed may account for noted decreases in germination rates.

25 B. Analysis of pCGN3242 Transformed *Brassica napus* cv. A112

A dramatic increase in stearate composition is observed in mature self-pollinated seeds of a transformed plant (3242-

4 A-3), from 1.8% to 39.8%. Increased stearate is accompanied by a decreased percentage of 18:1 and an increased percentage of ~~18:1~~, 18:3 and long chain saturated fatty acids. T2 seed from the 3242-A-3 plant yields a somewhat continuous range of percent stearate in individual seeds up to 45% stearate. Oil content of high stearate 3242-A112 seed is variable, some seeds having over 30% stearate also have an oil content comparable to control A112 seeds. Segregation and Southern analysis indicate that in 3242-A-3 three functional T-DNA inserts are seen. Independent segregation of multiple antisense genes displaying various levels of expression may account for the range of stearate levels observed.

C. Triacylglyceride Analysis of Mature Brassica Seeds (3242-Tobin1)

15 Mature T2 seeds of *Brassica campestris* cv. Tobin containing pCGN3242 are crushed and 250µg of C17:0 triglyceride in 250µg of toluene is added as an internal standard. The seeds are extracted with 1ml of a 3:2 hexane/isopropanol mixture, dried down, and resuspended in 20 100µl to make uniform solvent concentrations in each sample. 20µl of each sample is placed on a silica gel TLC plate (Baker-flex Silica Gel 1B2, 20X20 cm, 200µm thick) and run to the top with 100ml of a 60:40 hexane/diethyl ether/acetic acid solvent system. 50µg of standards containing tri-, di-, 25 and monoglycerides, as well as free fatty acid are run in adjacent lanes, and can be visualized by spraying these lanes with 10% <sup>phosphomolybdic</sup> ~~phosphomolybdic~~ acid in methanol. After heating the



spray lanes in an oven, these spots are used as a reference to cut out the non-visualized spots in the sample lane. The plant pieces containing TAG are extracted with 3ml of 3:2 hexane/ isopropanol, dried down and analyzed for fatty acid content by the acidic methanolysis method of Browse *et al.* (supra). The analyses demonstrates a triglycerol fatty acid composition substantially unchanged, including stearate content, from that observed in analysis of total seed lipids.

#### 10 D. Analysis of pCGN3234 Transformed Plants

Some abnormalities have been observed in some transgenic *Brassica napus* cv. Delta and Bingo and *Brassica campestris* cv. Tobin plants containing pCGN3234. Thus, preliminary results suggest that constitutively expressive antisense desaturase may <sup>interfere</sup> ~~interfer~~ with plant growth. These effects could be due to the constitutive expression of antisense desaturase RNA from the 35S promoter (i.e., perhaps providing undesirable leaf lipid compositions) or could be due to the transformation/tissue culture regime the plants have been subjected to, as examples.

The above results demonstrate the ability to obtain plant  $\Delta$ -9 desaturases, isolate DNA sequences which encode desaturase activity and manipulate them. In this way, the production of transcription cassettes, including expression cassettes can be produced which allow for production, including specially differentiated cell production of the

desired product. A purified *C. tinctorius* desaturase is provided and used to obtain nucleic acid sequences of *C. tinctorius* desaturase. Other plant desaturase sequences are provided such as *R. communis*, *B. campestris*, and *S.*  
5 *chinensis*. These sequences as well as desaturase sequences obtained from them may be used to obtain additional desaturase, and so on. And, as described in the application modification of oil composition may be achieved.

10 All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each  
15 individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain  
20 changes and modifications may be practiced within the scope of the appended claim.